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(71)出願人　390035404
グリュネンタール・ゲゼルシャフト・ミト・ベシュレンクテル・ハフツング
ドイツ連邦共和国、52078　アーヒエン、ツイーグレルストラーセ、６

(72)発明者　シユテフアン・ヴネント
ドイツ連邦共和国、52076　アーヒエン、フランドリッシエ・ストラーセ、18

(72)発明者　ゲルト・ヨーゼフ・シユテフエンス
ドイツ連邦共和国、52072　アーヒエン、ムッフエッター・ウエーク、50

(74)代理人　弁理士　江崎　光史　（外３名）

最終頁に続く

(54)【発明の名称】　繊維素溶解性のトロンビン阻害性質を有するキメラたん白質

(57)【要約】
【課題】　繊維素溶解性の血液凝固阻害性質を有するキメラたん白質。
【解決手段】　本発明はプラスミノーゲンを活性化するアミノ酸配列のＣ－最終末端でトロンビンを阻害するアミノ酸配列と結合する、繊維素溶解性の血液凝固阻害性質を有するキメラたん白質に関する。

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【特許請求の範囲】

【請求項１】 プラスミノーゲンを活性化するアミノ酸＊配列のＣ－最終末端で式Ｉ

【化１】

Ser-X_1-X_2-X_3-X_4-X_5-Pro-Arg-Pro-Y_1-Y_2-Y_3-Y_4-Asn-Pro-Z

（式中X_1はＰｒｏ又はＬｅｕ、X_2はＧｌｙ、Ｖａｌ又はＰｒｏ、X_3はＬｙｓ、Ｖａｌ、Ａｒｇ、Ｇｌｙ又はＧｌｕ、X_4はＡｌａ、Ｖａｌ、Ｇｌｙ、Ｌｅｕ又はＩｌｅ、X_5はＧｌｙ、Ｐｈｅ、Ｔｒｐ、Ｔｙｒ又はＶａｌ、Y_1はＰｈｅ、Ｔｙｒ又はＴｒｐ、Y_2はＬｅ※u、Ａｌａ、Ｇｌｙ、Ｉｌｅ、Ｓｅｒ又はＭｅｔ、Y_3はＬｅｕ、Ａｌａ、Ｇｌｙ、Ｉｌｅ、Ｓｅｒ又はＭｅｔ、Y_4はＡｒｇ、Ｌｙｓ又はＨｉｓ及びＺは式ＩＩ

【化２】

Gly-Asp-Z_1-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln

（式中Z_1はＰｈｅ又はＴｙｒを示す。）又は式ＩＩＩ★ ★【化３】

Asn-Asp-Lys-Tyr-Glu-Pro-Phe-Glu-Glu-Tyr-Leu-Gln

又は式ＩＶ

Ser-Asp-Phe-Glu-Glu-Phe-Ser-Leu-Asp-Asp-Ile-Glu-Gln

又はＶ

Ser-Glu-Phe-Glu-Glu-Phe-Glu-Ile-Asp-Glu-Glu-Glu-Lys

のアミノ酸配列を示す。）のアミノ酸配列と結合する、繊維素溶解性のトロンビン阻害性質を有するキメラたん白質。

【請求項２】 プラスミノーゲンを活性化するアミノ酸配列は、プロウロキナーゼの不変化アミノ酸配列；欠失、置換、挿入及び（又は）付加によって修飾されたプロウキナーゼのアミノ酸配列の少なくとも１種、ウロキナーゼの不変化アミノ酸配列；欠失、置換、挿入及び（又は）付加によって修飾されたウロキナーゼのアミノ酸配列少なくとも１種、組織プラスミノーゲン活性化因子（ｔ－ＰＡ）の不変化アミノ酸配列；欠失、置換、挿入及び（又は）付加によって修飾されたｔ－ＰＡのアミノ酸配列、コウモリ－プラスミノーゲン活性化因子（ｂａｔ－ＰＡ）の不変化アミノ酸配列；欠失、置換、挿入及び（又は）付加によって修飾されたｂａｔ－ＰＡのアミノ酸配列少なくとも１種及び（又は）ストレプトキナーゼ、スタフィロキナーゼ及び（又は）ＡＰＳＡＣのアミノ酸配列を含有する、請求項１記載のたん白質。

【請求項３】プラスミノーゲンを活性化するアミノ酸配列は、プロウロキナーゼの不変化アミノ酸配列；欠失、置換、挿入及び（又は）付加によって修飾されたプロウロキナーゼのアミノ酸配列少なくとも１種、ｔ－ＰＡの不変化アミノ酸配列及び（又は）欠失、置換、挿入及び（又は）付加によって修飾されたｔ－ＰＡのアミノ酸配列少なくとも１種を含有する、請求項２記載のたん白質。

【請求項４】 プラスミノーゲンを活性化するアミノ酸配列は、不変化の、アミノ酸４１１個から成るプロウロキナーゼの配列――これの中でアミノ酸は'' Ａｓｎ又はＧｌｎの位置にある――から、プロウロキナーゼのアミノ酸配列''Ｓｅｒ～ ''' Ｌｅｕ――これ中でアミノ酸は'' Ａｓｎ又はＧｌｎの位置にある――から、プロウロキナーゼのアミノ酸配列 '''Ｓｅｒ～ ''' Ｌｅｕ――これ中でアミノ酸は'' Ａｓｎ又はＧｌｎの位置にある――から、不変化の、アミノ酸５２７個から成るｔ－ＰＡのアミノ酸配列Ｓｅｒ－''Ａｒｇ～ ''' Ｐｒｏから及び（又は）ｔ－ＰＡのアミノ酸配列 '''Ｓｅｒ～ ''' Ｐｒｏから成る、請求項３記載のたん白質。

【請求項５】 式Ｉのアミノ酸配列中、X_1はＰｒｏ、X_2はＶａｌ、X_3はＬｙｓ又はＶａｌ、X_4はＡｌａ、X_5はＰｈｅを示す、請求項１ないし４のいずれかに記載のたん白質。

【請求項６】 式Ｉのアミノ酸配列中、Y_1はＰｈｅ、Y_2はＬｅｕ、Y_3はＬｅｕ、Y_4はＡｒｇを示す、請求項１ないし５のいずれかに記載のたん白質。

【請求項７】 式Ｉのアミノ酸配列中、Ｚは式ＩＩ又は式ＩＶのアミノ酸配列を示す、請求項１ないし６のいずれかに記載のたん白質。

【請求項８】 オペロンは制御可能なプロモーター、リボソーム結合部位として有効なシャイン・ダルガーノ配列、開始コドン、請求項１ないし７による繊維素溶解性質を有するたん白質に対する合成構造遺伝子及び構造遺伝子から上流へターミネーター１又は２個を有し、そしてプラスミドは、大腸菌株中で繊維素溶解性質を有するたん白質の発現に適する、請求項１ないし７のいずれかに記載の繊維素溶解性質を有するたん白質を産生するためのプラスミド。

【請求項９】 ｐＳＥ１及びｐＳＥ９より成る群から選ばれる、請求項８記載のプラスミド。

【請求項１０】　請求項８又は９に記載されたプラスミドを産生するにあたり、これを図１～１２に記載されたプラスミドｐＢｌｕｅ－ｓｋｒｉｐｔ　ＫＳＩＩ'、ｐＵＣ８及びｐＧＲ２０１から産生することを特徴とする上記プラスミドの産生方法。

【請求項１１】　プラスミドを用いて大腸菌－株を公知方法で形質転換し、構造遺伝子の発現を誘発し、生じたたんぱく質前駆体を培地及び溶解された細菌細胞から分離し、溶解し、次いでレドックス系の作用によって繊維素溶解性質を有するたん白質に折りたたむことを特徴とする、請求項１ないし７のいずれかに記載された繊維素溶解性質を有するたん白質の産生に請求項８又は９に記載されたプラスミドを使用する方法。

【請求項１２】　有効物質として請求項１ないし７のいずれかに記載のキメラたん白質を含有する血栓溶解剤。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】本発明は、繊維素溶解性の血液凝固阻害性質を有するキメラたん白質――これは、プラスミノーゲンを活性化するアミノ酸配列のＣ－最終末端でトロンビンを阻害するアミノ酸配列と結合する――、このポリペプチドを産生するためのプラスミド及び有効物質としてこの様なポリペプチドを含有する血栓溶解剤に関する。

【０００２】

【従来の技術】すべての工業国に於て、心臓－循環器系疾患が現在最も頻発する死因である。その際特に重要なことは、急性の血栓症閉塞を生じ、その発生が心筋梗塞の場合、最も短時間以内に心筋への生命に危険な供給不足を生じる。同様なことが脳梗塞にもいえる。この場合脳内閉塞は、病気に見舞われた脳領域の大きい虚血性損傷を伴う。高い死亡率と結びつく心筋梗塞に反して、脳梗塞に於ける供給不足は一般に急性の生命に危険な状態を導びかず、特定の脳機能の欠損によって日々の性格に著しい悪影響を与え、したがって一部、病気に見舞われた人の生活習慣の極端な欠陥を生じる。上記２つの梗塞形態は一般に、病気に見舞われた動脈によって供給される領域が数時間以内に――治療せずに――回復不能に傷つけられるとされている。治療を必要とする他の血栓症性閉塞疾患は、肺塞栓、静脈血栓症及び末梢の動物閉塞疾患である。

【０００３】血栓によって引き起こされる血管の閉塞は、ほとんど動脈硬化障害でフィブリン、血栓及び赤血球から血液凝固システムの種々の酵素の作用下に生じる。血液凝固システムの酵素カスケードのうち、トロンビンが重要な役割を果たす。トロンビンは血液凝固システムの重要な酵素すべてを活性化することができ、血小板の凝集を誘発し、フィブリノーゲンをフィブリンに変えることによってフィブリンクロット(Fibringespinte)の形成を生じる(Furie及びFurie, New Engl. J. Med,３２６，８００（１９９２））。

【０００４】血栓の形成は、生理学的血液凝固阻止剤、たとえばアンチトロンビンＩＩＩ、活性化されたたん白質Ｃ及び組織因子経路阻害剤によって制御される。一度形成された血栓を、生体に特有なプラスミンの作用によって再び溶解することができる。プラスミンは不活性なプロ酵素、プラスミノーゲンから生じ、このプラスミンはプラスミノーゲン活性化因子によってたん白質分解して活性化される。プラスミンによって引き起こされる血栓溶解は、血栓症疾患の患者、特に急性心筋梗塞の患者をプラスミノーゲン活性化因子で処置する治療に利用される。多くの治療が病気に見舞われた梗塞領域を縮小し、死亡を減少させる。現在この治療に、ストレプトキナーゼ、ＡＰＳＡＣ　(Anisolated Plasminogen Streptokinase Activator Complex)、二本鎖ウロキナーゼ（ＵＫ）、組換え型一本鎖ウロキナーゼ（組換え型プロウロキナーゼ）及び組織プラスミノーゲン活性化因子（ｔ－ＰＡ）が使用されている(Collen 及び Lijnen, Blood ７８，３１１４（１９９１））。溶解治療の従来存在する経験から、閉塞された冠状血管の再切開は梗塞の発作後数時間以内で、すなわち１～４時間で最良の機能連結果を生じることが明らかである。最適な再灌流（Reperfusion)の目的を達成するために、多くの場合に、治療開始は一定の吸収に先立って実際上始めねばならない。しかしこれはこの時点でまだ安全でない診断法を顧慮すれば副作用のあまりないかつ安全な繊維素溶解剤を用いてしか行われない。しかしいわゆる第一タイプのすべての繊維素溶解剤、たとえばストレプトキナーゼ、ＡＰＳＡＣ及びウロキナーゼは、急性梗塞の治療に於て必要な投薬で全身性プラスミノーゲン活性化を生じる。このことは、高い出血危険を伴う。いわゆる第二タイプの繊維素溶解剤、ｔ－ＰＡ及びプロウロキナーゼの使用は、多くの梗塞患者に於て系統的プラスミノーゲン活性化を生じる。再灌流を成果あるものにし、かつ再閉塞を回避するために、ｔ－ＰＡ及びプロウロキナーゼを明らかな繊維素溶解、すなわち系統的プラスミノーゲン活性化を生じる。高い投薬量で使用しなければならない。このことは、従来の研究に於て、出血性合併症の発生率の点でｔ－ＰＡ又はプロウロキナーゼで治療した患者とストレプトキナーゼで治療した患者の間の著しい相違は検出することができなかったという観察結果と一致する。　したがってプラスミノーゲン活性化因子の薬理学的特徴を改良するために、種々の試みが続けられた。

【０００５】更に、コウモリ－プラスミノーゲン活性化因子(Gardell等, J. Biol. Chem.２６４，１７９４７（１９８９）；ヨーロッパ特許第３８３４１７号明細書）、スタフィロキナーゼ(Schlott等、Bio／Technology １２，１８５（１９９４）；Collen及びVan De Werf, Circulation ８７，１８５０（１９９３））、組換え型組織プラスミノーゲン活性化因子ＢＭ　０６．０２

2(Martin 等、J. Cardiovasc. Pharm. 18, 111（1991））並びにt－PA－変異体のTNK-t－PA(Keyt 等、Proc. Natl. Acad. Sci.91, 3670（1994））が開発されている。

【0006】ストレプトキナーゼ、すなわち溶血性レンサ球菌のたん白質はヒトプラスミノーゲンを活性化する。というのはこのたん白質はプラスミノーゲンと複合体を生成し、それによってプラスミノーゲンが活性な構造に変化するからである。この複合体それ自体は遊離のプラスミノーゲンをプラスミンに変え、このプラスミンはその時再びストレプトキナーゼと結合するプラスミノーゲンを切断する。同様にスタフィロキナーゼ、すなわち黄色ブドウ球菌から得られるたん白質も作用するが、これはストレプトキナーゼに比してより高いフィブリン特異性を有する。ストレプトキナーゼが更に開発されたものは、APSAC、ストレプトキナーゼとヒトプラスミノーゲンから試験管内で産生された化合物である。APSACは、プラスミノーゲンの活性中心の化学修飾によってストレプトキナーゼに比して高められた生物学的半減期を有する。

【0007】ウロキナーゼは、ヒトのたん白質であり、これは2つの形態でたん白質分解に活性なたん白質としてウリンから得ることができる：高分子ウロキナーゼ（HUK）及び低分子ウロキナーゼ（LUK(Stump等、J. Biol. Chem. 261, 1267（1986））。HUK及びLUKは、ウロキナーゼの活性型、すなわち二本鎖分子である。ウロキナーゼを一本鎖ウロキナーゼ（プロウロキナーゼ）として種々の組織中に生成し、プロ酵素として少量でヒト血液中で検出することができる(Wun等、J. Biol. Chem. 257, 3276（1982））。プロウロキナーゼの活性型はHUKとして54キロダルトンの分子量を有し、3個のドメインから成る：アミノ－末端成長因子－ドメイン、クリングル及びセリン－プロテアーゼ－ドメイン(Quenzler 等、Hoppe-Seyler's Z. Physiol. Chem.363, 1155（1982）；Steffens等、Hoppe-Seyler's Z. Physiol. Chem. 363, 1043（1982））。プロウロキナーゼ及びプラスミノーゲンはプロ酵素として存在けれども、プロウロキナーゼは内的活性のゆえにプラスミノーゲンを活性プラスミンに変えることができる。しかしこのプラスミノーゲン活性化因子は、生成されたプラスミンそれ自体がプロウロキナーゼを ^{135}リジン及び ^{136}イソロイシンの間で切断した後に初めて十分な活性を維持する(Lijnen 等、J. Biol.Chem. 261, 1253（1986））。大腸菌中でウロキナーゼを遺伝子工学で産生する方法は、Heyneker等によって初めて記載された(Proceedings of the IVth International Symposium on Genetics of Industrial Microorganisms 1982）。非グリコシル化されたプロウロキナーゼ(Saruplase）を合成遺伝子の使用下に生成する(Brigelius-Flohe等、Appl.

Microbiol. Biotech. 36, 640（1992））。

【0008】t－PAは、血液中及び組織中に産生する、分子量72キロダルトンのたん白質である。このプラスミノーゲン活性化因子は5個のドメインから成る：アミノ－末端フィンガードメイン、成長－因子－ドメイン、クリングル一、クリングル2及びセリン－プロテアーゼ－ドメイン。プロウロキナーゼの様に、t－PAをクリングル2とセリン－プロテアーゼ－ドメインの間、すなわち ^{275}Argと^{276}Ileの間でプラスミン触媒による切断によって活性な二本鎖型に変える。試験管内試験及び動物実験による結果によればt－PAはフィブリンと結合し、そしてその酵素活性はフィブリンによって刺激されることが示されている(Collen 及び Lijnen, Blood 78, 3114（1991））。t－PAのフィブリン特異性によって、プラスミンが血液系全体中で生成され、後にフィブリンばかりでなく、フィブリノーゲンも分解するのを回避しなければならない。この様な系統的なプラスミノーゲン活性化並びにフィブリノーゲンの著しい分解は望ましいものではない。というのはこれが出血の危険を高めるからである。いずれにせよ治療実務で、前臨床実験から生じる、t－PAのフィブリン特異性に関する期待は、満たされないことが明らかである。また上述の様にt－PAの短い生物学的半減期のために、高い投薬量を注入しなければならない。この投薬量はフィブリン特異性にもかかわらず系統的プラスミノーゲンの活性化を生じる(Keyt等、Proc. Natl. Acad. Sci. 91, 3670（1994））。

【0009】r－PA及びTNK－t－PAは、改良された性質を有するt－PA－変異体である。r－PA（BM06.022）の場合、最初の3つのt－PA－ドメイン、すなわちフィンガードメイン、成長因子－ドメイン及び第一クリングルを欠失するので、短くなった分子は第二クリングルとプロテアーゼドメインしか含有しない。r－PAは遺伝子工学で大腸菌中に産生され、グリコシル化されない。t－PAに比してr－PAはより長い生物学的半減期を有し、より急速な再循流を生じる。動物実験で、ボルスとして投与されるr－PAはt－PA－注入と同様に有効であることが明らかである(Martin 等、J. Cardiovasc. Pharmacol. 18, 111（1991））。

【0010】t－PA－変異体TNK－t－PAは、次の3つの点で天然t－PAと相違する：^{103}トレオリンをアスパラギンと交換、それによって新しいグリコシル化部位を生じる；^{117}アスパラギンをグルタミンと交換、それによってグリコシル化部位を除き、^{296}リジンと^{299}アルギニンの間の配列を4個の連続するアラニン－単位と交換する。これらの3つの変異の組合せは、天然t－PAに比してより高いフィブリン特異性及びより長い生物学的半減期を有するポリペプチドを生じる。更に、TNK－t－PAは、天然t－PAに比して著しく

急速にＰＡＩ－１によって阻害される(Keyt 等、Proc. Natl. Acad. Sci. 91, 3670（1994））。ＴＮＫ－ｔ－ＰＡの前駆体を用いて得られる動物実験の結果は、ＴＮＫ－ｔ－ＰＡがボルス投与に適していることを示している(Refino 等、Thromb. Haemost. 70、313（1993））。

【００１１】コウモリプラスミノーゲン活性化因子(bat-PA)は、吸血コウモリ(FledermausDesmodus rotundus)のつば中に見い出される。遺伝子工学で産生されたこれらのプラスミノーゲン活性化因子は、ｔ－ＰＡよりも更に優れたフィブリン特異性を有し、動物実験で、高められた生物学的半減期及び減少された系統的プラスミノーゲン活性化と共に改良された血栓溶解を示す(Gardell 等、Circulation 84, 244（1991））。

【００１２】血栓症疾患の治療に於て、プラスミノーゲン活性化因子を一般に血液凝固阻止物質、たとえばヘパリンと一緒に投与する。それによってプラスミノーゲン活性化因子での単独処理に於けるよりも改良された血栓溶解が得られる(Tebbe等、Z.Kardiol. 80, Suppl. 3, 32（1991））。臨床から得られた種々の所見は、血栓の溶解に平行して高められた血液凝固傾向を生じることを示している(Szczeklik等、Arterioscl. Thromb. 12, 548（1992）；Goto等、Angiology 45, 273（1994））。このことには血栓中に包含され、血餅の溶解の際に再び遊離されるトロンビン分子が原因になっていると考えられる。更に、プラスミノーゲン活性化因子それ自体もプロトロンビンの活性化を促進し、それ故に血栓溶解を押さえるはたらきをすることが示されている（Brommer 及びMeijer, Thromb. Haemostas.70, 995（1993））。血液凝固阻止物質、たとえばヘパリン、ヒルゲン、ヒルジン、アルガトロバン、プロテインＣ及び組換え型ダニ血液凝固阻止ペプチド（ＴＡＰ）は、血栓溶解の間、強められた再閉塞傾向を阻止し、それ故に溶解治療の結果を改良する(Yao等、Am. J. Physiol. 262（HEART Circ. Physiol.31）H347－H379（1992）；Schneider, Theomb. Res. 64, 667（1991）；Gruber等、Circulation84, 2454（1991）；Martin等、J. Am. Coll. Cardiol. 22, 914（1993）；Vlasuk等、Circulation 84, Suppl.ＩＩ－467（1991）。

【００１３】強められたトロンビン阻害剤の１つは、６５個のアミノ酸から成る、チスイビル(Hirudo medicinales)から得られるヒルジンである。いくつかのアミノ酸が異なる種々のヒルジン－同型が得られる。すべてのヒルジン－同型はトロンビンと物質、たとえばフィブリノーゲンとの結合及びトロンビンの活性中心を遮断する(Rydel等、Science 249, 277（1990）；Bode及びHuber, MolecularAspects of Inflammation, スプリンガー、ベルリン、ハイデルベルク, 103－115（1991）；Stone 及び Hofsteenge, Prot. Enfineering 2, 295（1991）；Dodt等、Biol. Chem. Hoppe-Seyler 366, 379（1985）．更にヒルジンから生じる小さい分子は公知であり、これは同様にトロンビン阻害活性を有する(Maraganore 等、Biochemistry 29, 7095（1990）；Krstenansky 等、J. Med. Chem. 30, 1688（1987）；Yue 等、Prot. Engineering 5, 77（1992））。

【００１４】ヒルジンをプラスミノーゲン活性化因子と組合せて、血栓症疾患に使用することは、ヨーロッパ特許第３２８９５７号及び第３６５４６８号明細書中に記載されている。ヒルジン誘導体を血栓溶解剤と組合せて使用することは、国際特許出願ＷＯ９１／０１１４２から公知である。ヒルリンはチスイビル(Hirudo manillensis)から単離された６１個のアミノ酸を有するたん白質である。ヒルリンはその作用及び阻害強度の点でヒルジンと同等であるが、アミノ酸配列の点でヒルジンと著しく異なる。ヒルリンから、トロンビンを極めて良好に阻害するより小さい分子を生じることもできる(Krstenansky等、Febs Lett. 269, 465（1990））。

【００１５】更に、トロンビンを、ヒトトロンビンレセプターのアミノ－末端配列から生じるペプチドによって阻害することもできる(Vu 等、Nature 253, 674（1991））。このトロンビンレセプターは、細胞外のアミノ－末端域で隣接する切断部位を有するトロンビン結合配列をトロンビンに対して有する。この配列は、切断部位を41セリンから42フェニルアラニンに交換することによってマスクする場合、トロンビンを阻害することができる。

【００１６】Phaneuf 等は Thromb. Haemost. 71, 481（1994）に、ストレプトキナーゼ及びヒルジンの偽発性化学結合から成る複合体が記載されている。しかしプラスミノーゲンを活性化する能力は、このストレプトキナーゼ－ヒルジン－複合体の場合非修飾のストレプトキナーゼの下で因子８あたりにある。

【００１７】

【発明を解決しようとする課題】本発明による課題は、極めて短い時間内で完全な血栓溶解を生じさせ、同時に血管の再度の閉塞を、まず第一に有効な血栓溶解後に阻害する、血栓症が原因の血管閉塞を治療するための有効物質を開発することである。更に、この有効物質を用いて系統的プラスミノーゲン活性化因子を回避しなければならない。

【００１８】

【問題を解決するための手段】本発明者は、プラスミノーゲンを活性化するアミノ酸配列のＣ－最終末端に、トロンビンを阻害するアミノ酸配列を有する有効物質に対して求められる高い要求が繊維素溶解性質を有するキメラたん白質によってかなえられることを見い出した。

【００１９】したがって、本発明の対象はプラスミノーゲンを活性化するアミノ酸配列のＣ－最終末端で式Ｉ

【0020】 ＊ ＊【化4】

Ser-X_1-X_2-X_3-X_4-X_5-Pro-Arg-Pro-Y_1-Y_2-Y_3-Y_4-Asn-Pro-Z

【0021】（式中X_1はＰｒｏ又はＬｅｕ、X_2はＧｌｙ、Ｖａｌ又はＰｒｏ、X_3はＬｙｓ、Ｖａｌ、Ａｒｇ、Ｇｌｙ又はＧｌｕ、X_4はＡｌａ、Ｖａｌ、Ｇｌｙ、Ｌｅｕ又はＩｌｅ、X_5はＧｌｙ、Ｐｈｅ、Ｔｒｐ、Ｔｙｒ又はＶａｌ、Y_1はＰｈｅ、Ｔｙｒ又はＴｒｐ、Y_2はＬｅｕ、Ａｌａ、Ｇｌｙ、Ｉｌｅ、Ｓｅｒ又は※ ※はＭｅｔ、Y_3はＬｅｕ、Ａｌａ、Ｇｌｙ、Ｉｌｅ、Ｓｅｒ又はＭｅｔ、Y_4はＡｒｇ、Ｌｙｓ又はＨｉｓ及びＺは式ＩＩ

Gly-Asp-Z_1-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln

【0022】

【0023】（式中Z_1はＰｈｅ又はＴｙｒを示す。） ★【0024】

【化5】

又は式ＩＩＩ ★

【化6】

Asn-Asp-Lys-Tyr-Glu-Pro-Phe-Glu-Glu-Tyr-Leu-Gln

又は式ＩＶ

Ser-Asp-Phe-Glu-Glu-Phe-Ser-Leu-Asp-Asp-Ile-Glu-Gln

又はＶ

Ser-Glu-Phe-Glu-Glu-Phe-Glu-Ile-Asp-Glu-Glu-Glu-Lys

【００２５】のアミノ酸配列を示す。）のアミノ酸配列と結合する、繊維素溶解性のトロンビン阻害性質を有するキメラたん白質である。本発明によるキメラたん白質は、式Ｉのトロンビン－阻害アミノ酸配列を介してトロンビンと結合する。それによって血餅に於てキメラたん白質の高い濃度が達成される。急性心筋梗塞又は脳梗塞で生じる血餅はトロンビンが豊富であるので、本発明によるたん白質の血栓特異性は、プラスミノーゲン活性化因子の血栓溶解有効性及び選択性を高める可能性を提供する。これによって系統的プラスミノーゲン活性化及び繊維素溶解を回避し、有効物質の安全性が明らかに増加する。血栓特異性によって投薬量も慣用のプラスミノーゲン活性化因子に比して減少する。このことは調製物の安全性も増加させる。同時に抗凝固性共薬剤（たとえばヘパリン含有）の投薬量は本発明によるたん白質の使用で減少することが予想される。更に抗凝固剤の追加は必要でなくなる。

【００２６】好ましいキメラたん白質は、プラスミノーゲンを活性化するアミノ酸配列は、プロウロキナーゼの不変化アミノ酸配列；欠失、置換、挿入及び（又は）付加によって修飾されたプロウキナーゼのアミノ酸配列の少なくとも１種、ウロキナーゼの不変化アミノ酸配列；欠失、置換、挿入及び（又は）付加によって修飾されたウロキナーゼのアミノ酸配列少なくとも１種、組織プラスミノーゲン活性化因子（ｔ－ＰＡ）の不変化アミノ酸配列；欠失、置換、挿入及び（又は）付加によって修飾されたｔ－ＰＡのアミノ酸配列、コウモリ－プラスミノーゲン活性化因子（ｂａｔ－ＰＡ）の不変化アミノ酸配列；欠失、置換、挿入及び（又は）付加によって修飾されたｂａｔ－ＰＡのアミノ酸配列少なくとも１種及び（又は）ストレプトキナーゼ、スタフィロキナーゼ及び（又は）ＡＰＳＡＣのアミノ酸配列を含有する。

【００２７】プラスミノーゲンを活性化するアミノ酸配列は、プロウロキナーゼの不変化アミノ酸配列；欠失、置換、挿入及び（又は）付加によって修飾されたプロウロキナーゼのアミノ酸配列少なくとも１種、ｔ－ＰＡの不変化アミノ酸配列及び（又は）欠失、置換、挿入及び（又は）付加によって修飾された、ｔＰＡのアミノ酸配列少なくとも１種を含有する。プラスミノーゲンを活性化するアミノ酸配列が、不変化の、アミノ酸４１１個から成るプロウロキナーゼの配列――これの中でアミノ酸は'' Ａｓｎ又はＧｌｎの位置にある――から、プロウロキナーゼのアミノ酸配列'' Ｓｅｒ～ ''' Ｌｅｕ――これ中でアミノ酸は'' Ａｓｎ又はＧｌｎの位置にある――から、プロウロキナーゼのアミノ酸配列 ''' Ｓｅｒ～''' Ｌｅｕ ――これ中でアミノ酸は'' Ａｓｎ又はＧｌｎの位置にある――から、不変化の、アミノ酸５２７個から成るｔ－ＰＡのアミノ酸配列Ｓｅｒ－'' Ａｒｇ～ ''' Ｐｒｏから及び（又は）ｔ－ＰＡのアミノ酸配列 ''' Ｓｅｒ～''' Ｐｒｏから成るたん白質が特に好ましい。

【００２８】キメラたん白質に於て、プラスミノーゲンを活性化するアミノ酸配列はＣ－最終末端で好ましくは式Ｉのトロンビンを阻害するアミノ酸配列（式中X_1はＰｒｏ、X_2はＶａｌ、X_3はＬｙｓ又はＶａｌ、X_4はＡｌａ、X_5はＰｈｅを示す。）と結合する。式Ｉのアミノ酸配列中、Y_1は好ましくはＰｈｅ、Y_2は好ましくはＬｅｕ、Y_3は好ましくはＬｅｕ、Y_4は好まし

くはＡｒｇを示す。式Ｉのアミノ酸配列中、変化可能なZは、特に式ＩＩ又はＩＶのアミノ酸配列を示す。

【００２９】公知のプラスミノーゲン活性化因子；プラスミノーゲン活性化因子とトロンビン阻害剤とから成る公知の混合物並びに公知のストレプトキナーゼ－ヒルジン－複合体に比して、本発明によるたん白質は良好にトロンビンを阻害する性質と共に、強められた繊維素溶解作用の点で優れている。更に本発明によるポリペプチドによってプラズマフィブリノーゲンが明らかにより僅かな量で消費される。これから結果として生じるフィブリン特異性、特にプラスミノーゲン活性化因子とトロンビン阻害剤との公知の混合物と比較しても著しく高められたフィブリン特異性は次のことを生じさせる。それは血液の凝固性にほんの僅かにしか影響を与えず、そして制御されていない出血の危険を系統的フィブリノーゲン分解の起こりうる併発症として最も少なくすることである。したがって本発明によるたん白質の高いフィブリン特異性は、公知の血栓溶解剤のボルス投与に比して明らかに減少された出血の危険を有するボルス投与を可能にする。

【００３０】したがってもう１つの本発明の対象は、本発明によるたん白質を有効物質として含有する血栓溶解剤である。血栓症の原因となる血管閉塞、たとえば心筋梗塞、脳梗塞、末梢の急性動脈閉塞、肺塞栓、不安定な狭心症及び深在性下肢－及び骨盤静脈血栓症に、本発明によるポリペプチド０．１～１ｍｇ／ｋｇが必要である。本発明によるたん白質は静脈内にボルス注射又は注入によって投与される。

【００３１】本発明による血栓溶解剤は、少なくとも１種の本発明によるポリペプチドの他に助剤、たとえば賦形剤、溶剤、希釈剤、染料及び結合剤を含有する。これらの助剤の選択並びにその使用すべき量それ自体は、薬剤が投与されてよいこと、及び薬剤が当業者に問題なく調製されることに依る。本発明によるたん白質の産生は、遺伝子工学の方法によって行われる。更に、対応する遺伝子を、合成オリゴヌクレオチドから適当なプラスミド中に定着し、これはｔｒｐ－又はｔａｃ－プロモーターの制御下、特にｔｒｐ－プロモーターの制御下に大腸菌中で発現する。

【００３２】したがって本発明の対象は、本発明によるキメラたん白質を産生する際に使用するプラスミドにもあり、そのオペロンは制御可能なプロモーター、リボソーム結合部位として有効なシャイン・ダルガーノ配列、開始コドン、本発明によるたん白質に対する合成構造遺伝子及び構造遺伝子から上流へターミネーター１又は２個を有する。

【００３３】本発明によるプラスミドの発現を、大腸菌株、特にグループＫ１２の大腸菌、たとえばＥ．ｃｏｌｉ　Ｋ１２　ＪＭ１０１（ＡＴＣＣ３３８７６）、Ｅ．ｃｏｌｉ　Ｋ１２　ＪＭ１０３（ＡＴＣＣ３９４０３）、Ｅ．ｃｏｌｉ　Ｋ１２ＪＭ１０５（ＤＳＭ４１６２）及びＥ．ｃｏｌｉ　Ｋ１２　ＤＨ１（ＡＴＣＣ３３８４９）中で行う。細菌細胞中に、本発明によるポリペプチドは、たん白質が変性された形で存在する封入体の形で高収率で生じる。封入体の単離後、変性されたたん白質をたん白質化学でレドックス系の作用下所望の三次構造に折りたたむ。

【００３４】

【実施例】次に例によって本発明を説明する。

〔例１〕本発明によるたん白質の産生、単離及び精製。

ａ）定着処理

大腸菌中で本発明によるポリペプチドを遺伝子工学による産生に使用される発現プラスミドを公知の方法で生成する。各生成工程の順序を図１～２に示す。プラスミド生成の出発物は、プラスミド pBlueskript ＫＳＩＩ＋（Stratagene社、ハイデルベルグ）、ｐＵＣ８及びｐＳＬ１１９０（Pharmacia 社、フライブルグ）並びにｐＧＲ２０１である。ｐＧＲ２０１は、ヨーロッパ特許第４０８９４５号明細書及び Appl. Microbiol. Biotechn. 36，640－649（１９９２）に記載されているプラスミドｐＢＦ１８０と同一である。制限エンドヌクレアーゼBan II，BamHI，ClaI，HindIII，NcoI，NdeI，NheI及びNotI，並びにＤＮＡ－修飾された酵素、たとえばアルカリ性ホスファターゼ、Ｔ４－リガーゼ、Ｔ４－キナーゼ及びＴ７－ポリメラーゼを Pharmacia社、Stratagene社、Boehringer Mannheim及び Gibco（エグシュタイン）から入手する。その生成の間のプラスミドの変化を、制限分析及びＤＮＡ－配列分析によって検査する。ＤＮＡ-配列分析を製造者の説明書に従って Pharmacia社の試薬コレクションを用いて行う。プラスミドの生成にあたり、種々のオリゴデスオキシリボヌクレオチド(Oligos)を使用し、その配列を、関連する表示と共に表１中に記載する。

【００３５】オリゴデスオキシリボヌクレオチドを、脱トリチル化された形で０．１μＭｏｌ－スケールで、工場で適用されるバイオシステム(Weitersadt)の合成酵素（モデル３９１）を用いて製造者の説明書に従ってβ－シアノエチル－保護されたジイソプロピルアミノホスホアミジトの使用下に行う。夫々１００ｐｍｏｌのオリゴデスオキシリボヌクレオチドを、５０ｍＭトリ（ヒドロキシメチル）アミノメタン／ＨＣｌ（トリス／ＨＣｌ）、１０ｍＭ塩化マグネシウム及び５ｍＭジチオスレイトール中で７．５のｐＨ－値で酵素単位Ｔ４－キナーゼを用いて１０ｍＭアデノシントリホスフアートの存在下にホスホリル化し、次いで同一の緩衝液中で二本鎖ＤＮＡ－分子に変える。得られた合成の二本鎖ＤＮＡ－分子をゲル電気泳動によってポリアクリルアミドゲル（５％ポリアクリルアミド）上で精製し、次いで前もって調製されたプラスミドとの連結反応に使用する。制限酵素で消化、対応する制限フラグメントの単離及び５'-末端

の脱ホスホリル化、E．coli K12 JM103中での引き続きの連結反応及び形質転換並びにすべての他の遺伝子工学処理によるプラスミドの前もっての調製は、公知方法で行われ、Sambrook等、"Molecular Cloning: A Laboratory Manual", 2. Auflage, ColdSpring Harbor Laboratory Press, Cold Spring Harbour, アメリカ，1989中に記載されている。

【0036】

【表1】

オリゴ	5'からの配列を示す
O 105	TATGAGCAAAACTTGCTACGAAGGTAACGGTCACTTCTACCGTGGTAA GGCTTCTACCGACAC
O 106	CATGGTGTCGGTAGAAGCCTTACCACGGTAGAAGTGACCGTTACCTTC GTAGCAAGTTTTGGTCA
O 220	CGGTTAAGGCTTTCCCGAGGCCTGGTGGTGGTGGTAACGGTGACTTCG AAGAAATCCCGGAAGAGTACCTGTGATAGBATCAA
O 221	CTAGTTGATCCTATCACAGGTACTCTTCCGGGATTTCTTCGAAGTCAC CGTTACCACCACCACCAGGCCTCGGGAAAGCCTTAACCGGGCT
O 265	CACCCGGCGGAAGNCGGCGGGCTCAGNGCCAGNACCGTTTTCTTCTTTGGT GTGAGAACG
O 281	CGTCCGGGTGGTGGTGGTAACGGTGACTTCGAAGAAATCCCGGAAGAA TACCTGTAAG
O 282	GATCCGTTCTCACACCAAAGAAGAAAACGGTCTGGCTCTGNGCCCGCC GTCTCCGCCGGGTGGTTTCCCG
O 283	CTAGCTTACAGGTATTCTTCCGGGATTTCTTCGAAGTCACCGTTACCAC CACCACCCGGNCGCGGGAAAC
O 329	AAGAAATCCCGGAAGAATACCTGCAATAAG
O 330	CGGTTAAGGCTTGGGGACCGCGCCGCTGGGTGGTGGTGGTAACGGTGA CTTCG
O 331	ACCACCACCCAGCGCCGCGGTCCCCAAGCCTTAACCGGGCT
O 332	CTAGCTTATTGCAGGTATTCTTCCGGGATTTCTTCGAAGTCACCGTTAC C
O 347	CGGTTGTTGCTTTCCCGC
O 348	GGCCGCGGGAAAGCAACAACCGCGCT
O 545	CTAGCTTATTGCAGGTATTCTTCGAACGGGTCGTATTTGTCGTTAGGG TTACGCAGCAGGAAA
O 546	GGCCTTTCCTGCTGCGTAACCCTAACGACAAATACGAACCGTTCGAAG AATACCTGCAATAAC
O 615	CTAGCTTATTGCAGGTATTCTTCCGGGATTTCTTCGAAGTCACCAGGG TTACGCAGCAGGAAA
O 618	GGCCTTTCCTGCTGCGTAACCCTGGTGACTTCGAAGAAATCCCGGAAG AATACCTGCAATAAG

【0037】b）連続培養及び発酵の調製。

組換え型発現プラスミドpSE1（M38）及びpSE9（37）大腸菌 K12 JM103（ATCC39403）中に入れ、標準- I - 栄養寒天（MERCK 社、アンピシリン150mg／l）上に塗布する（Sambrook等、"MolecularCloning: A Laboratory Manual"）。各形質転換の単一コロニーの夫々を、標準- I - 栄養ブイヨン(Merck社、pH7．0；アンピシリン150mg／l）中で20℃で578nmで1の光学密度（OD）まで培養し、連続培養物として2ml づつ分けてジメチルスルホキシド（DMSO）（7．5%最終濃度）の添加下に－70℃で凍結し、保存する。本発明によるポリペプチドの産生のために、各連続培養物の夫々1mlを標準- I - 栄養ブイヨン20ml（pH7．0；150mg／lアンピシリン）中で懸濁し、37℃で578nmで1のODまで培養する。

【0038】次いで得られた培養物の全量を標準- I - 栄養ブイヨン11（pH7．0；150mg／lアンピシリン）中に懸濁し、振盪フラスコ中で37℃で発酵する。誘発はインドールアクリル酢酸溶液2ml（エタノール2ml中に60mg）の添加によって578nmで0．5～1のODで行われる。

c）発現テスト

発現度をテストするために、誘発の直前及び誘発後の各時間（全体で6時間）で、578nmで1のODを有する細胞懸濁液1mlに相当する細胞を遠心分離する。沈降された細胞を、リゾチーム（50mMトリス／HCl - 緩衝液、pH8．0、50mMエチレンジアミンテトラ酢酸（EDTA）及び15%サッカロース中でmlあたりリゾチーム1mg）を用いて加水分解する。溶解された細胞のホモジネートを、4～5Mグアニジニウムハイドロクロライド溶液中に溶解し、1．2Mグアニジニウムハイドロクロライドに希釈後、還元剤（グルタチオン又はシスティン）の添加下に2～5時間折りたたみ反

応を行う(Winkler等、Biochemistry　２５　４０４１～４０４５（１９８６））。得られた一本鎖の本発明によるポリペプチドを、プラスミンの添加によって対応する二本鎖分子に変え、その活性を色素産生基質ｐｙｒｏ－Ｇｌｕ－Ａｒｇ－ｐ－ニトロアニリドを用いて測定する。本発明によるポリペプチドのプラスミンによる活性化は、５０ｍＭトリス／ＨＣｌ－緩衝液、１２ｍＭ塩化ナトリウム、０．０２％トウィーン８０中でｐＨ７．４及び３７℃で行われる。本発明によるポリペプチドとプラスミンの割合は、酵素単位あたり約８，０００－３６，０００である。試験培養は、５０ｍＭトリス／ＨＣｌ－緩衝液及び３８ｍＭ塩化ナトリウム中でｐＨ８．８で０．３６μＭアプロチニン（プラスミンの阻害のために）及び０．２７ｍＭ基質ｐｙｒｏ－Ｇｌｕ－Ｇｌｙ－Ａｒｇ－ｐ－ニトロアニリドの存在下に３７℃で行われる。本発明によるポリペプチド濃度に関係なく、反応を５～６０分培養後５０％酢酸の添加によって停止し、４０５ｎｍで吸光を測定する。基質の製造者(Kadi Vitrum, スウェーデン）の説明書によれば、この処理で４０５ｎｍで１分あたり０．０５の吸光変化は、試験溶液１ｍｌあたり２５プラーク－単位のウロキナーゼ－活性に相当する。本発明によるポリペプチドは、たん白質１ｍｇあたり１２０，０００～１５５，０００プラーク－単位の比活性を有する。溶液のたん白質含有量は、Pierce社のＢＣＡ－検定法を用いて測定する。

ｄ）単離及び精製

６時間後、１ｂ）に記載された条件で行われた発酵を終了し（５７．８ｎｍで密度５～６ＯＤ）、細胞を遠心分離によって収得する。細胞沈降物を水２００ｍｌで再懸濁し、高圧ホモジナイザー中で分解する。新たに遠心分離した後、一本鎖の本発明によるポリペプチドの全量を含有する沈澱を、５Ｍグアニジニウムハイドロクロライド５００ｍｌ、４０ｍＭシステイン、１ｍＭ　ＥＤＴＡ中にｐＨ－値８．０で溶解し、ｐＨ－値９．０の２５ｍ＊Ｍトリス／ＨＣｌ　２０００ｍｌを用いて希釈する。折りたたみ反応を約１２時間後に完了する。

【００３９】得られた本発明によるポリペプチドを、シリカゲル８ｇの添加後２時間の攪拌によって完全にシリカゲルと結合する。結合されたシリカゲルを分離し、酢酸塩－緩衝液（ｐＨ４．０）で洗滌する。ポリペプチドを、０．５Ｍトリメチルアンモニウムクロライド（ＴＭＡＣ）を用いて０．１Ｍ酢酸塩－緩衝液（ｐＨ４）溶離する。２つのクロマトグラフィー分離（銅－キレート－カラムとカチオン交換体）後、ポリペプチドが純粋な形で得られる。Ｎ－末端配列分析によって一本鎖を確認する。

【００４０】すべての単離された本発明によるポリペプチド――そのアミノ酸配列は図１３～１４に記載されている――は、ウロキナーゼに対する色素産生基質を用いる直接的な活性度試験で全く又は極めて僅かしか活性度を示さない（１％以下）。プラスミンで分解した後に初めて（条件は１ｃの項に記載した）完全な酵素活性が得られる。したがって本発明によるポリペプチドは、大腸菌Ｋ１２　ＪＭ１０３中で一本鎖たん白質として発現する。

２．トロンビン阻害作用の測定

本発明によるポリペプチドの阻害活性を、ヒトクエン酸プラズマをベロナール緩衝液中でトロンビン溶液５０μｌ（０．２単位）で１：１０に希釈されたプラズマ２００μｌと本発明によるポリペプチド０．４～３０μｇを含有する水性溶液５０μｌを混合することによるトロンビン存続期間の測定によって決定する。表２中に記載されたトロンビン存続期間をプロウロキナーゼ又は本発明によるたん白質Ｍ３７及びＭ３８の存在下に測定する。プロウロキナーゼに反してＭ３７及びＭ３８は投薬量に関係なくトロンビン存続期間を延長し、したがって血液凝固の阻害剤として作用する

表　２：

	トロンビン存続期間〔秒〕		
たん白質	プロウロキナーゼ	Ｍ３７	Ｍ３８
０	３１	３２	３２
０．４		４０	
０．８		７９	
１．２		１４８	
１．６		１９５	
２．０		２６６	
４．０	３１	＞３００	５８
８．０			８１
１２．０			１０４
１６．０			１３０
２０．０	３３		１５０
３０．０	３３		＞３００

【図面の簡単な説明】

【図１】繊維素溶解性質を有するたん白質を生成するた

めのプラスミドを産生する方法を示す。

【図２】繊維素溶解性質を有するたん白質を生成するためのプラスミドを産生する方法を示す。

【図３】繊維素溶解性質を有するたん白質を生成するためのプラスミドを産生する方法を示す。

【図４】繊維素溶解性質を有するたん白質を生成するためのプラスミドを産生する方法を示す。

【図５】繊維素溶解性質を有するたん白質を生成するためのプラスミドを産生する方法を示す。

【図６】繊維素溶解性質を有するたん白質を生成するためのプラスミドを産生する方法を示す。

【図７】繊維素溶解性質を有するたん白質を生成するためのプラスミドを産生する方法を示す。

【図８】繊維素溶解性質を有するたん白質を生成するた＊

＊めのプラスミドを産生する方法を示す。

【図９】繊維素溶解性質を有するたん白質を生成するためのプラスミドを産生する方法を示す。

【図１０】繊維素溶解性質を有するたん白質を生成するためのプラスミドを産生する方法を示す。

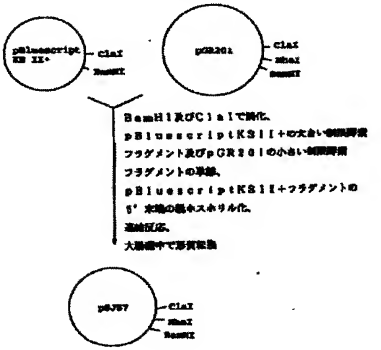
【図１１】繊維素溶解性質を有するたん白質を生成するためのプラスミドを産生する方法を示す。

【図１２】繊維素溶解性質を有するたん白質を生成するためのプラスミドを産生する方法を示す。

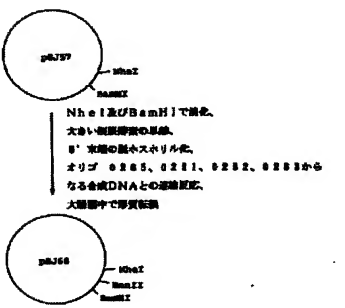
【図１３】本発明によるポリペプチドのアミノ酸配列を示す。

【図１４】本発明によるポリペプチドのアミノ酸配列を示す。

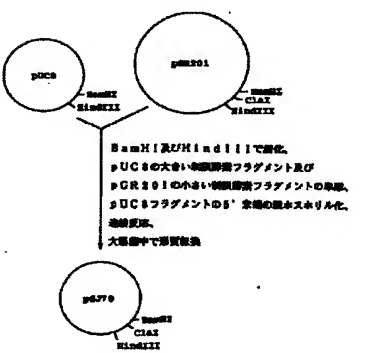
【図１】



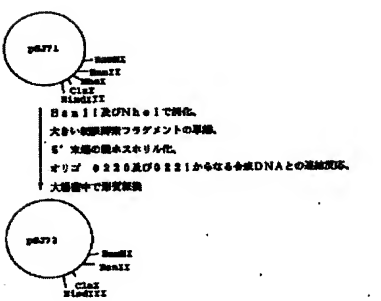
【図２】



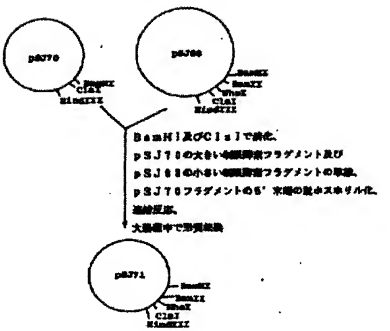
【図３】



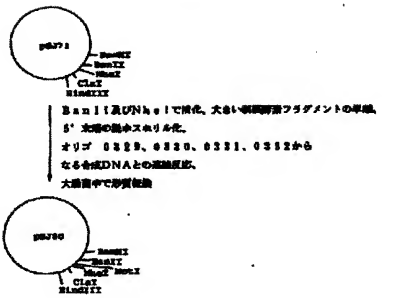
【図５】



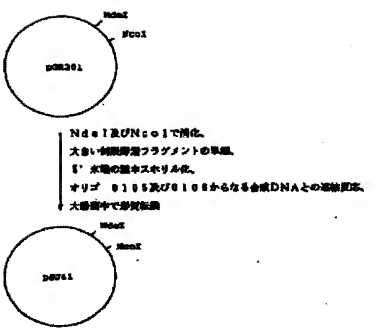
【図4】


pSJ70
pSJ66
BamHI
ClaI
HindIII
BamHI及びClaIで消化、
pSJ70の大きい制限酵素フラグメント及び
pSJ66の小さい制限酵素フラグメントの単離、
pSJ70フラグメントの5'末端の脱ホスホリル化、
連結反応、
大腸菌中で形質転換
pSJ71

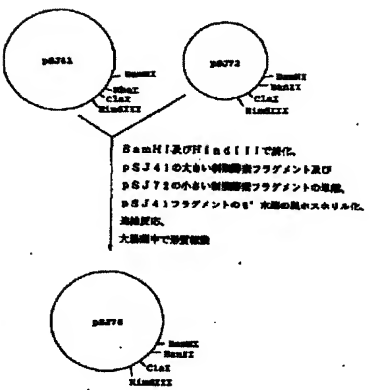
【図6】


pSJ71
BamHI
BamHI
XhoI
ClaI
HindIII
BamHI及びXhoIで消化、大きい制限酵素フラグメントの単離、
5'末端の脱ホスホリル化、
オリゴ　0329、0330、0331、0332から
なる合成DNAとの連結反応、
大腸菌中で形質転換
pSJ66

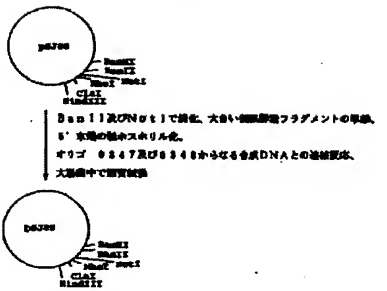
【図7】


NdeI
NcoI
pSE201
NdeI及びNcoIで消化、
大きい制限酵素フラグメントの単離、
5'末端の脱ホスホリル化、
オリゴ　0185及び0186からなる合成DNAとの連結反応、
大腸菌中で形質転換
NdeI
NcoI
pSJ51

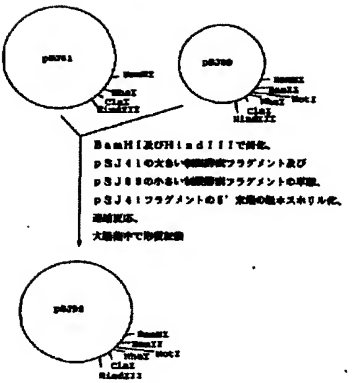
【図8】


pSJ41
pSJ72
BamHI
XhoI
ClaI
HindIII
BamHI及びHindIIIで消化、
pSJ41の大きい制限酵素フラグメント及び
pSJ72の小さい制限酵素フラグメントの単離、
pSJ41フラグメントの5'末端の脱ホスホリル化、
連結反応、
大腸菌中で形質転換
pSJ76

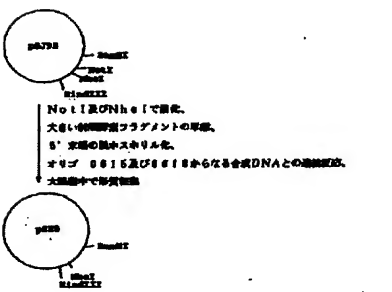
【図９】



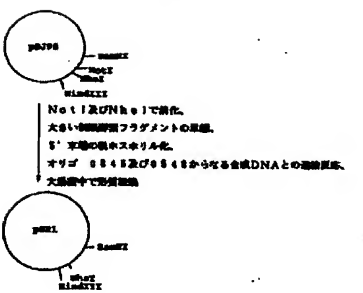
【図１０】



【図１１】



【図１２】



【図１３】

Abbildung 13: 図１３： Ｍ３７のアミノ酸配列

Met-Ser-Lys-Thr-Cys-Tyr-Glu-Gly-Asn-Gly-His-Phe-Tyr-Arg-
Gly-Lys-Ala-Ser-Thr-Asp-Thr-Met-Gly-Arg-Pro-Cys-Leu-Pro-
Trp-Asn-Ser-Ala-Thr-Val-Leu-Gln-Gln-Thr-Tyr-His-Ala-His-
Arg-Ser-Asp-Ala-Leu-Gln-Leu-Gly-Leu-Gly-Lys-His-Asn-Tyr-
Cys-Arg-Asn-Pro-Asp-Asn-Arg-Arg-Arg-Pro-Trp-Cys-Tyr-Val-
Gln-Val-Gly-Leu-Lys-Pro-Leu-Val-Gln-Glu-Cys-Met-Val-His-
Asp-Cys-Ala-Asp-Gly-Lys-Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu-
Leu-Lys-Phe-Gln-Cys-Gly-Gln-Lys-Thr-Leu-Arg-Pro-Arg-Phe-
Lys-Ile-Ile-Gly-Gly-Glu-Phe-Thr-Thr-Ile-Glu-Asn-Gln-Pro-
Trp-Phe-Ala-Ala-Ile-Tyr-Arg-Arg-His-Arg-Gly-Gly-Ser-Val-
Thr-Tyr-Val-Cys-Gly-Gly-Ser-Leu-Ile-Ser-Pro-Cys-Trp-Val-
Ile-Ser-Ala-Thr-His-Cys-Phe-Ile-Asp-Tyr-Pro-Lys-Lys-Glu-
Asp-Tyr-Ile-Val-Tyr-Leu-Gly-Arg-Ser-Arg-Leu-Asn-Ser-Asn-
Thr-Gln-Gly-Glu-Met-Lys-Phe-Glu-Val-Glu-Asn-Leu-Ile-Leu-
His-Lys-Asp-Tyr-Ser-Ala-Asp-Thr-Leu-Ala-His-His-Asn-Asp-
Ile-Ala-Leu-Leu-Lys-Ile-Arg-Ser-Lys-Glu-Gly-Arg-Cys-Ala-
Gln-Pro-Ser-Arg-Thr-Ile-Gln-Thr-Ile-Cys-Leu-Pro-Ser-Met-
Tyr-Asn-Asp-Pro-Gln-Phe-Gly-Thr-Ser-Cys-Glu-Ile-Thr-Gly-
Phe-Gly-Lys-Glu-Asn-Ser-Thr-Asp-Tyr-Leu-Tyr-Pro-Glu-Gln-
Leu-Lys-Met-Thr-Val-Val-Lys-Leu-Ile-Ser-His-Arg-Glu-Cys-
Gln-Gln-Pro-His-Tyr-Tyr-Gly-Ser-Glu-Val-Thr-Thr-Lys-Met-
Leu-Cys-Ala-Ala-Asp-Pro-Gln-Trp-Lys-Thr-Asp-Ser-Cys-Gln-
Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-Cys-Ser-Leu-Gln-Gly-Arg-
Met-Thr-Leu-Thr-Gly-Ile-Val-Ser-Trp-Gly-Arg-Gly-Cys-Ala-
Leu-Lys-Asp-Lys-Pro-Gly-Val-Tyr-Thr-Arg-Val-Ser-His-Phe-
Leu-Pro-Trp-Ile-Arg-Ser-His-Thr-Lys-Glu-Glu-Asn-Gly-Leu-
Ala-Leu-Ser-Pro-Val-Val-Ala-Phe-Pro-Arg-Pro-Phe-Leu-Leu-
Arg-Asn-Pro-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-
Gln

【図14】

Abbildung 14: 図14： M38のアミノ酸配列

Met-Ser-Lys-Thr-Cys-Tyr-Glu-Gly-Asn-Gly-His-Phe-Tyr-Arg-
Gly-Lys-Ala-Ser-Thr-Asp-Thr-Met-Gly-Arg-Pro-Cys-Leu-Pro-
Trp-Asn-Ser-Ala-Thr-Val-Leu-Gln-Gln-Thr-Tyr-His-Ala-His-
Arg-Ser-Asp-Ala-Leu-Gln-Leu-Gly-Leu-Gly-Lys-His-Asn-Tyr-
Cys-Arg-Asn-Pro-Asp-Asn-Arg-Arg-Arg-Pro-Trp-Cys-Tyr-Val-
Gln-Val-Gly-Leu-Lys-Pro-Leu-Val-Gln-Glu-Cys-Met-Val-His-
Asp-Cys-Ala-Asp-Gly-Lys-Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu-
Leu-Lys-Phe-Gln-Cys-Gly-Gln-Lys-Thr-Leu-Arg-Pro-Arg-Phe-
Lys-Ile-Ile-Gly-Gly-Glu-Phe-Thr-Thr-Ile-Glu-Asn-Gln-Pro-
Trp-Phe-Ala-Ala-Ile-Tyr-Arg-Arg-His-Arg-Gly-Gly-Ser-Val-
Thr-Tyr-Val-Cys-Gly-Gly-Ser-Leu-Ile-Ser-Pro-Cys-Trp-Val-
Ile-Ser-Ala-Thr-His-Cys-Phe-Ile-Asp-Tyr-Pro-Lys-Lys-Glu-
Asp-Tyr-Ile-Val-Tyr-Leu-Gly-Arg-Ser-Arg-Leu-Asn-Ser-Asn-
Thr-Gln-Gly-Glu-Met-Lys-Phe-Glu-Val-Glu-Asn-Leu-Ile-Leu-
His-Lys-Asp-Tyr-Ser-Ala-Asp-Thr-Leu-Ala-His-His-Asn-Asp-
Ile-Ala-Leu-Leu-Lys-Ile-Arg-Ser-Lys-Glu-Gly-Arg-Cys-Ala-
Gln-Pro-Ser-Arg-Thr-Ile-Gln-Thr-Ile-Cys-Leu-Pro-Ser-Met-
Tyr-Asn-Asp-Pro-Gln-Phe-Gly-Thr-Ser-Cys-Glu-Ile-Thr-Gly-
Phe-Gly-Lys-Glu-Asn-Ser-Thr-Asp-Tyr-Leu-Tyr-Pro-Glu-Gln-
Leu-Lys-Met-Thr-Val-Val-Lys-Leu-Ile-Ser-His-Arg-Glu-Cys-
Gln-Gln-Pro-His-Tyr-Tyr-Gly-Ser-Glu-Val-Thr-Thr-Lys-Met-
Leu-Cys-Ala-Ala-Asp-Pro-Gln-Trp-Lys-Thr-Asp-Ser-Cys-Gln-
Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-Cys-Ser-Leu-Gln-Gly-Arg-
Met-Thr-Leu-Thr-Gly-Ile-Val-Ser-Trp-Gly-Arg-Gly-Cys-Ala-
Leu-Lys-Asp-Lys-Pro-Gly-Val-Tyr-Thr-Arg-Val-Ser-His-Phe-
Leu-Pro-Trp-Ile-Arg-Ser-His-Thr-Lys-Glu-Glu-Asn-Gly-Leu-
Ala-Leu-Ser-Pro-Val-Val-Ala-Phe-Pro-Arg-Pro-Phe-Leu-Leu-
Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-Glu-Glu-Tyr-Leu-
Gln

フロントページの続き

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C12R 1:19)				

(72)発明者 エルケ・ヤノカ
ドイツ連邦共和国、52441 リンニッヒ、ヘールストラーセ、65

(72)発明者 レジナ・ハインツエルーウィーラント
ドイツ連邦共和国、64295 ダルムシュタット、ライヒエンベルゲル・ウエーク、16

Ref. 5

US006133011A

United States Patent [19]

Wnendt et al.

[11] Patent Number: 6,133,011

[45] Date of Patent: Oct. 17, 2000

[54] **CHIMERIC PROTEINS HAVING FIBRINOLYTIC AND THROMBIN-INHIBITING PROPERTIES**

[75] Inventors: **Stephan Wnendt**; **Gerd Josef Steffens**, both of Aachen; **Elke Janocha**, Linnich; **Regina Heinzel-Wieland**, Darmstadt, all of Germany

[73] Assignee: **Gruenenthal GmbH**, Aachen, Germany

[21] Appl. No.: **08/967,024**

[22] Filed: **Nov. 10, 1997**

Related U.S. Application Data

[63] Continuation of application No. 08/563,649, Nov. 28, 1995.

[30] **Foreign Application Priority Data**

Nov. 30, 1994 [DE] Germany 44 42 665

[51] **Int. Cl.**[7] **A61K 38/36**; C12N 9/72; C12N 15/58; C12N 15/62

[52] **U.S. Cl.** **435/212**; 435/69.7; 435/252.3; 435/252.33; 435/320.1; 424/94.64; 536/23.4

[58] **Field of Search** 435/69.6, 212, 435/252.3, 255.33, 320.1, 69.7; 424/94.64; 536/23.4

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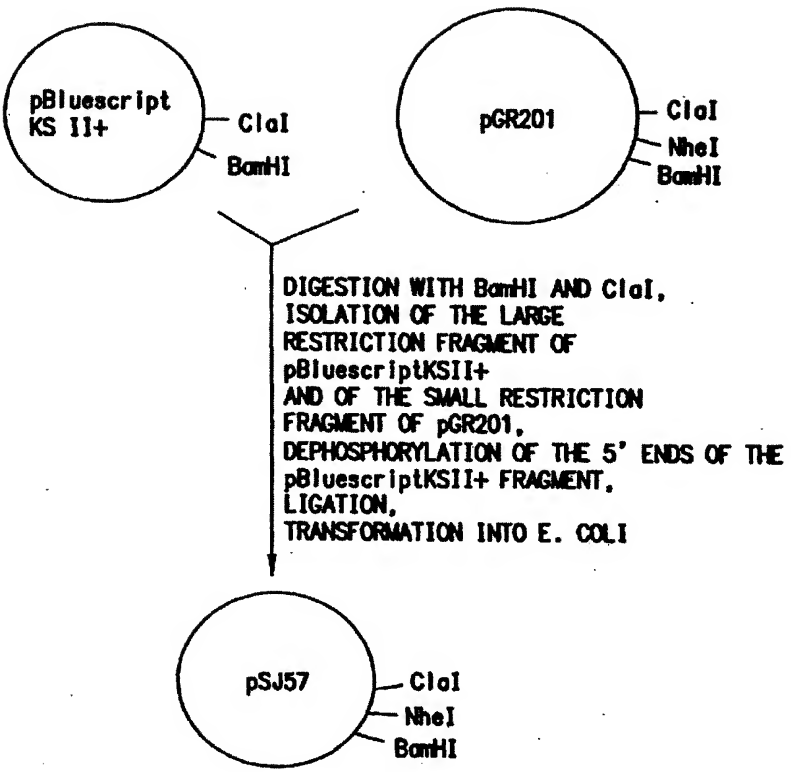
Primary Examiner—Ponnathapu Achutamurthy
Assistant Examiner—William W. Moore
Attorney, Agent, or Firm—Evenson, McKeown, Edwards & Lenahan, P.L.L.C.

[57] **ABSTRACT**

Chimeric proteins with fibrinolytic and thrombin-inhibiting properties having a plasminogen-activating amino acid sequence which is linked at its C-terminal end to a thrombin-inhibiting amino acid sequence.

7 Claims, 14 Drawing Sheets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KS II+
ClaI
BamHI
pGR201
ClaI
NheI
BamHI
DIGESTION WITH BamHI AND ClaI,
ISOLATION OF THE LARGE
RESTRICTION FRAGMENT OF
pBluescriptKSII+
AND OF THE SMALL RESTRICTION
FRAGMENT OF pGR201,
DEPHOSPHORYLATION OF THE 5' ENDS OF THE
pBluescriptKSII+ FRAGMENT,
LIGATION,
TRANSFORMATION INTO E. COLI
pSJ57
ClaI
NheI
BamHI

FIG.1

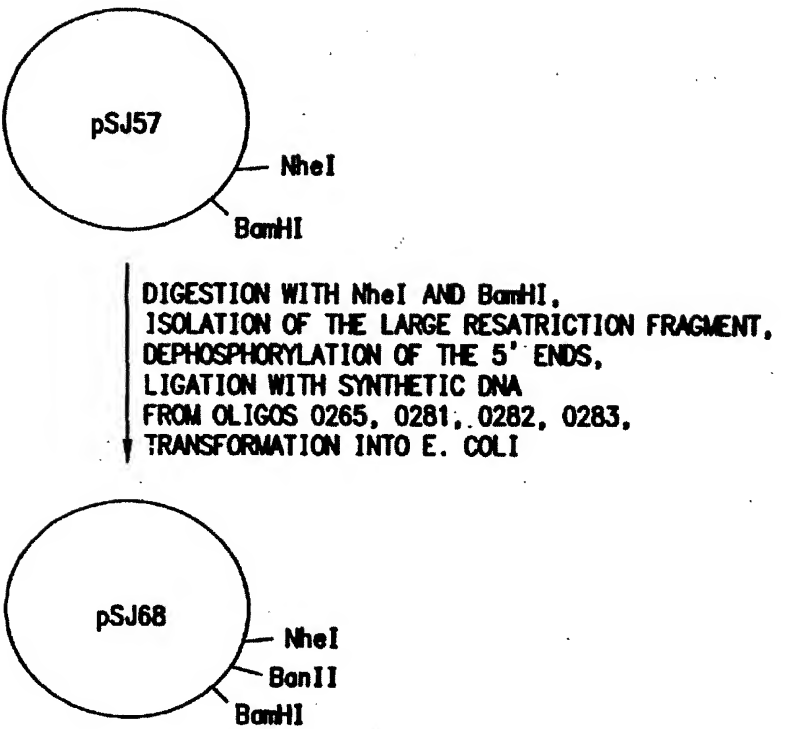

pSJ57
NheI
BamHI
DIGESTION WITH NheI AND BamHI,
ISOLATION OF THE LARGE RESATRICTION FRAGMENT,
DEPHOSPHORYLATION OF THE 5' ENDS,
LIGATION WITH SYNTHETIC DNA
FROM OLIGOS 0265, 0281, 0282, 0283,
TRANSFORMATION INTO E. COLI
pSJ68
NheI
BanII
BamHI

FIG.2

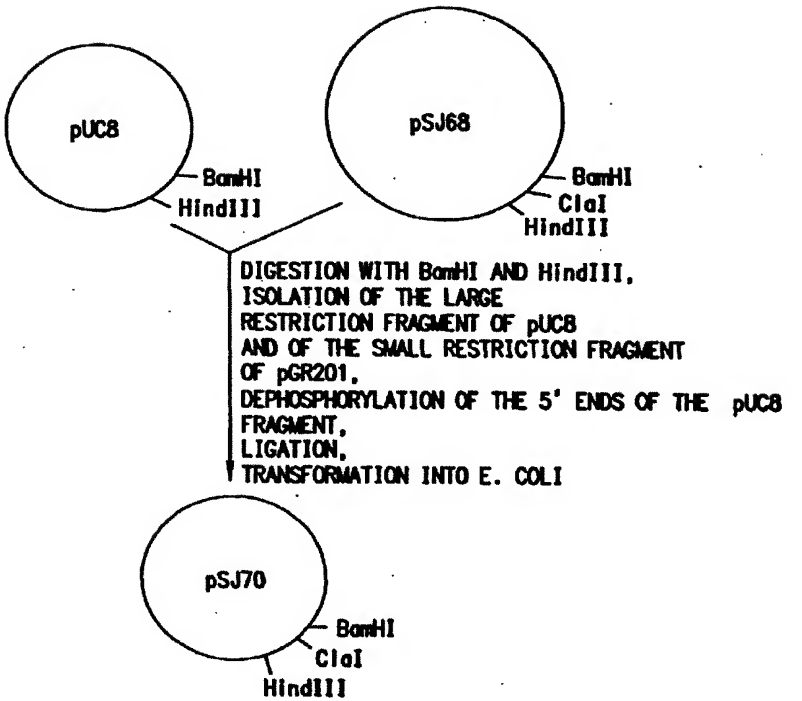

pUC8
BamHI
HindIII
pSJ68
BamHI
ClaI
HindIII
DIGESTION WITH BamHI AND HindIII,
ISOLATION OF THE LARGE
RESTRICTION FRAGMENT OF pUC8
AND OF THE SMALL RESTRICTION FRAGMENT
OF pGR201,
DEPHOSPHORYLATION OF THE 5' ENDS OF THE pUC8
FRAGMENT,
LIGATION,
TRANSFORMATION INTO E. COLI
pSJ70
BamHI
ClaI
HindIII

FIG.3

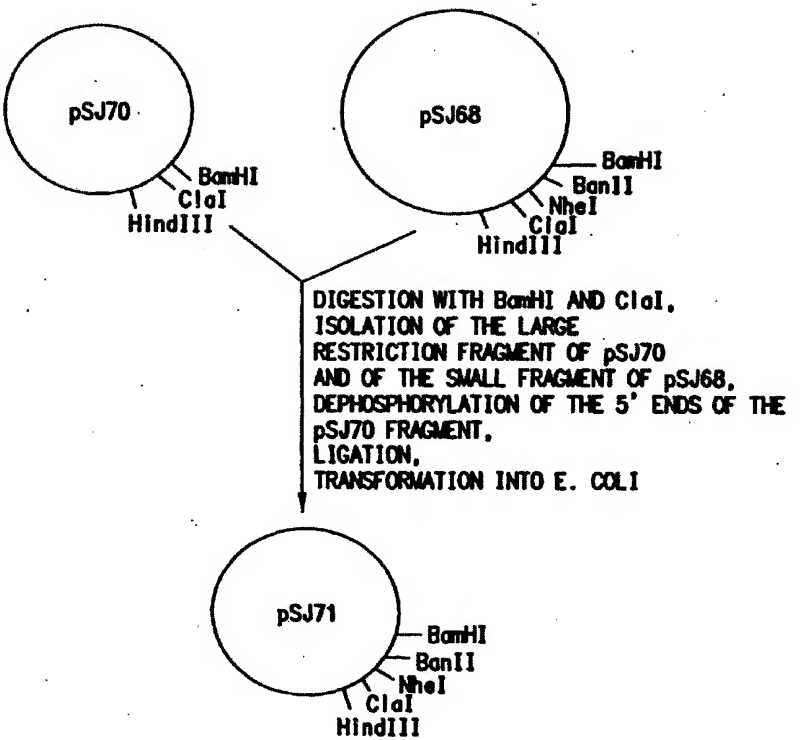

pSJ70
BamHI
ClaI
HindIII
pSJ68
BamHI
BanII
NheI
ClaI
HindIII
DIGESTION WITH BamHI AND ClaI,
ISOLATION OF THE LARGE
RESTRICTION FRAGMENT OF pSJ70
AND OF THE SMALL FRAGMENT OF pSJ68,
DEPHOSPHORYLATION OF THE 5' ENDS OF THE
pSJ70 FRAGMENT,
LIGATION,
TRANSFORMATION INTO E. COLI
pSJ71
BamHI
BanII
NheI
ClaI
HindIII

FIG.4

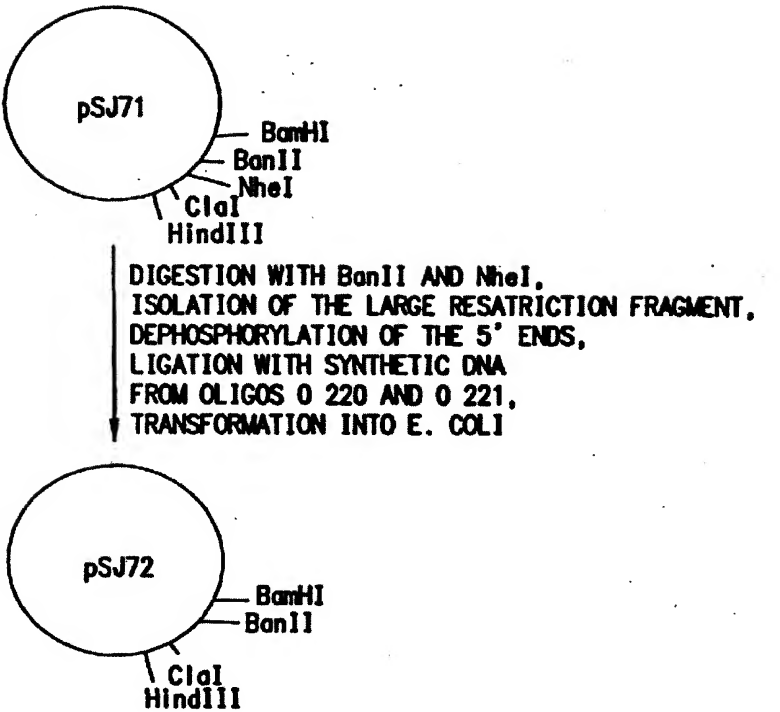

pSJ71
BamHI
BanII
NheI
ClaI
HindIII
DIGESTION WITH BanII AND NheI,
ISOLATION OF THE LARGE RESATRICTION FRAGMENT,
DEPHOSPHORYLATION OF THE 5' ENDS,
LIGATION WITH SYNTHETIC DNA
FROM OLIGOS 0 220 AND 0 221,
TRANSFORMATION INTO E. COLI
pSJ72
BamHI
BanII
ClaI
HindIII

FIG.5

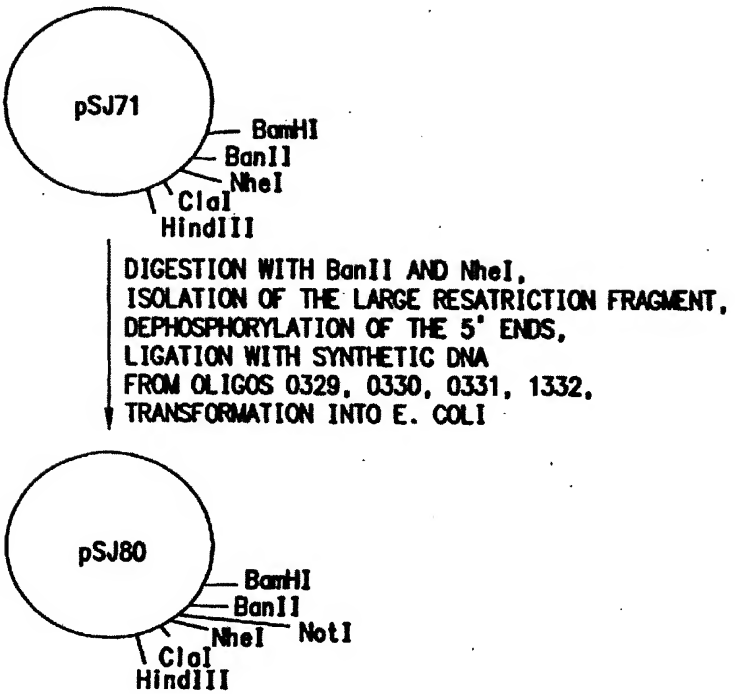

pSJ71
BamHI
BanII
NheI
ClaI
HindIII
DIGESTION WITH BanII AND NheI,
ISOLATION OF THE LARGE RESATRICTION FRAGMENT,
DEPHOSPHORYLATION OF THE 5' ENDS,
LIGATION WITH SYNTHETIC DNA
FROM OLIGOS 0329, 0330, 0331, 1332,
TRANSFORMATION INTO E. COLI
pSJ80
BamHI
BanII
NheI
NotI
ClaI
HindIII

FIG.6

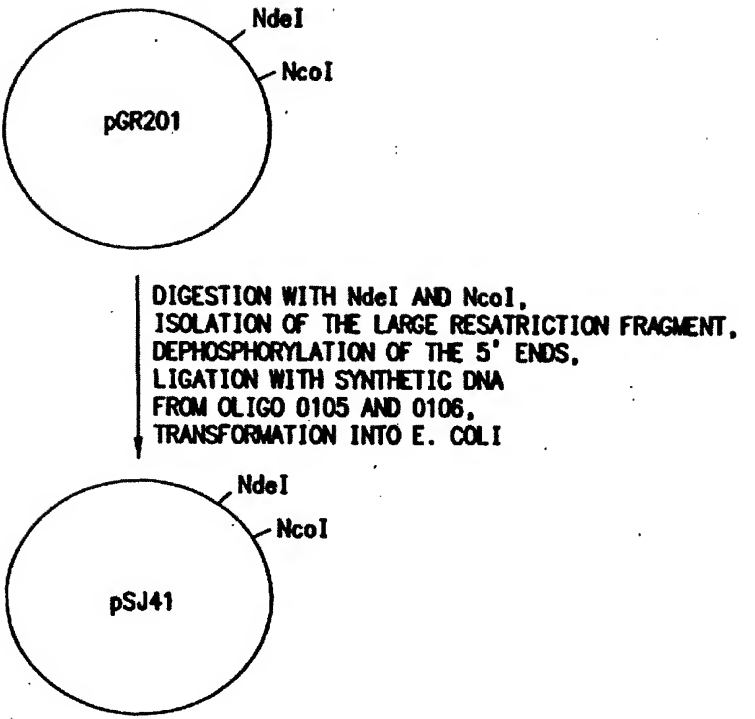

NdeI
NcoI
pGR201
DIGESTION WITH NdeI AND NcoI,
ISOLATION OF THE LARGE RESATRICTION FRAGMENT,
DEPHOSPHORYLATION OF THE 5' ENDS,
LIGATION WITH SYNTHETIC DNA
FROM OLIGO 0105 AND 0106,
TRANSFORMATION INTO E. COLI
NdeI
NcoI
pSJ41

FIG.7

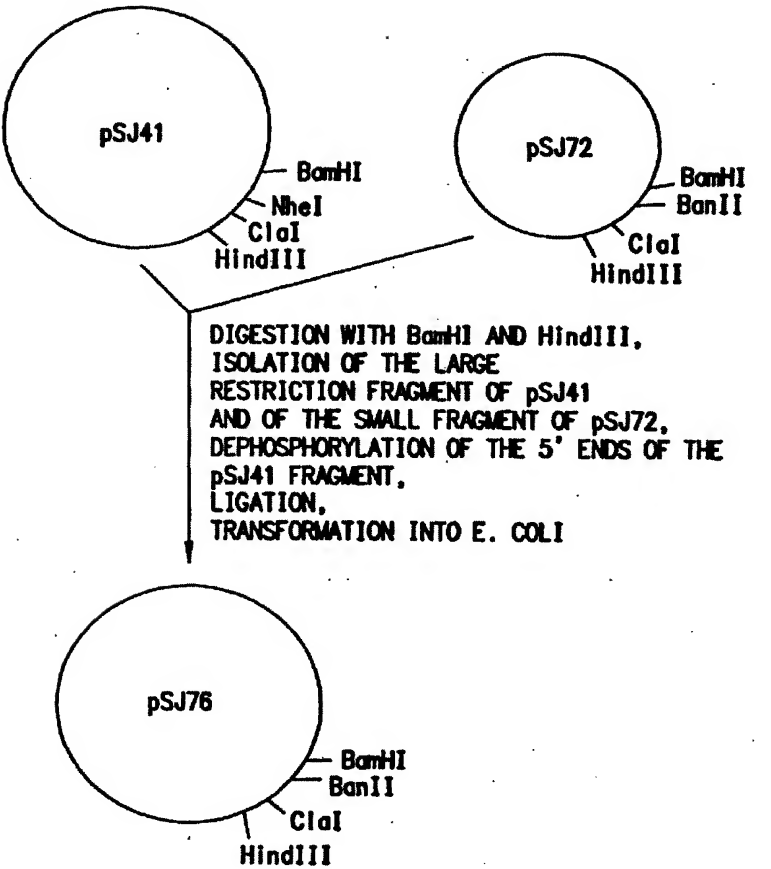

pSJ41
BamHI
NheI
ClaI
HindIII
pSJ72
BamHI
BanII
ClaI
HindIII
DIGESTION WITH BamHI AND HindIII,
ISOLATION OF THE LARGE
RESTRICTION FRAGMENT OF pSJ41
AND OF THE SMALL FRAGMENT OF pSJ72,
DEPHOSPHORYLATION OF THE 5' ENDS OF THE
pSJ41 FRAGMENT,
LIGATION,
TRANSFORMATION INTO E. COLI
pSJ76
BamHI
BanII
ClaI
HindIII

FIG.8

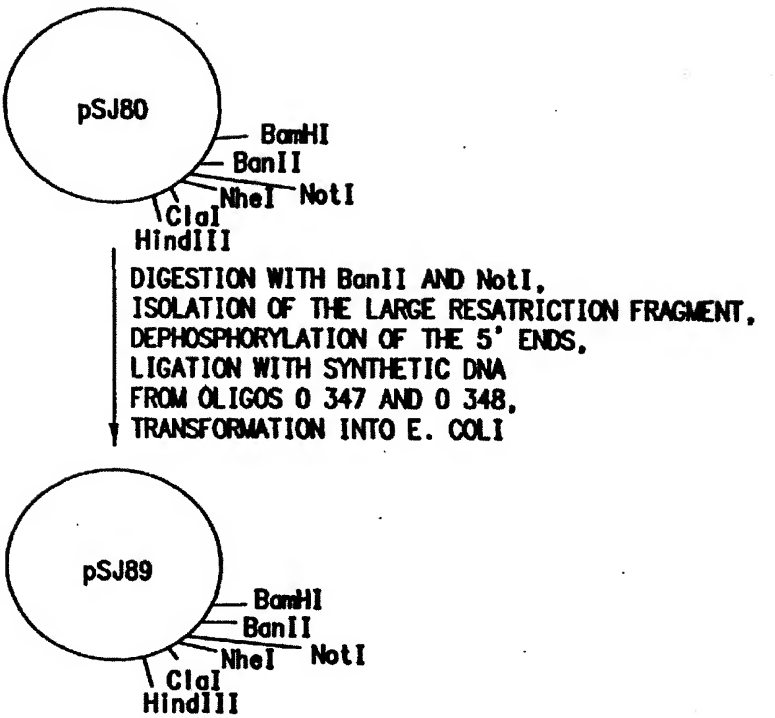
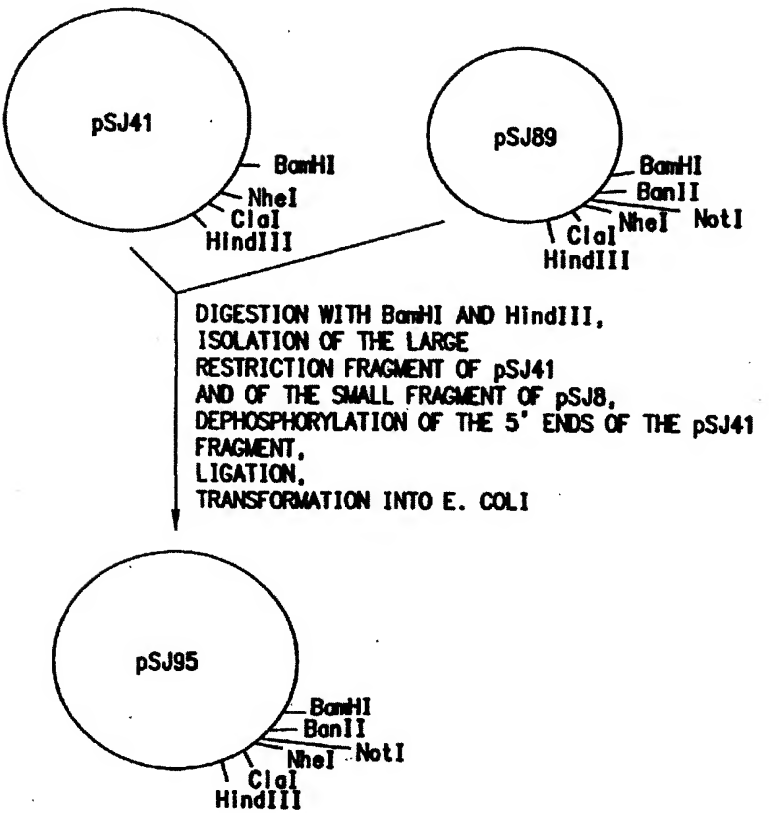

pSJ80
BamHI
BanII
NheI
NotI
ClaI
HindIII
DIGESTION WITH BanII AND NotI,
ISOLATION OF THE LARGE RESATRICTION FRAGMENT,
DEPHOSPHORYLATION OF THE 5' ENDS,
LIGATION WITH SYNTHETIC DNA
FROM OLIGOS O 347 AND O 348,
TRANSFORMATION INTO E. COLI
pSJ89
BamHI
BanII
NheI
NotI
ClaI
HindIII

FIG.9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BamHI
NheI
ClaI
HindIII
pSJ89
BamHI
BanII
NheI
NotI
ClaI
HindIII
DIGESTION WITH BamHI AND HindIII,
ISOLATION OF THE LARGE
RESTRICTION FRAGMENT OF pSJ41
AND OF THE SMALL FRAGMENT OF pSJ8,
DEPHOSPHORYLATION OF THE 5' ENDS OF THE pSJ41
FRAGMENT,
LIGATION,
TRANSFORMATION INTO E. COLI
pSJ95
BamHI
BanII
NheI
NotI
ClaI
HindIII

FIG.10

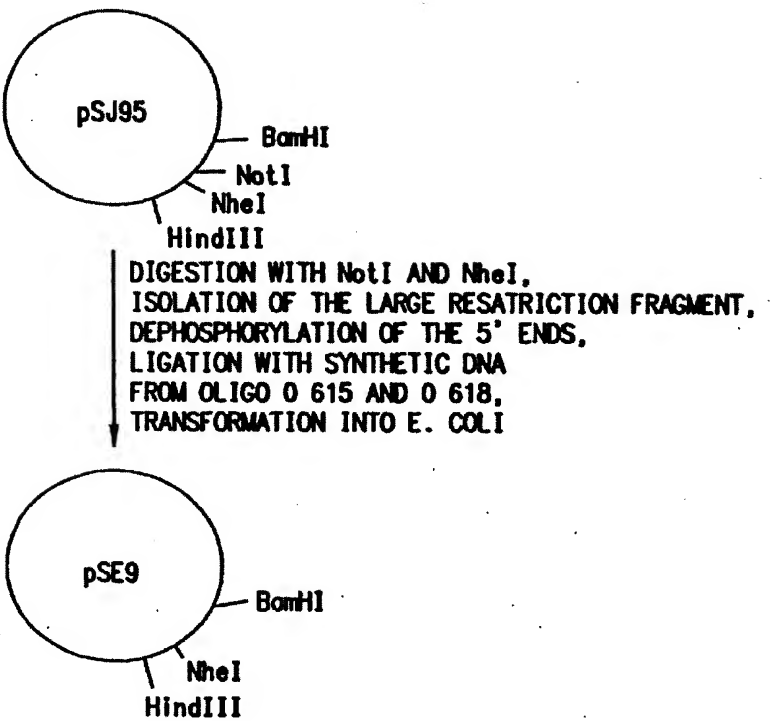
pSJ95
BamHI
NotI
NheI
HindIII
DIGESTION WITH NotI AND NheI,
ISOLATION OF THE LARGE RESATRICTION FRAGMENT,
DEPHOSPHORYLATION OF THE 5' ENDS,
LIGATION WITH SYNTHETIC DNA
FROM OLIGO 0 615 AND 0 618,
TRANSFORMATION INTO E. COLI
pSE9
BamHI
NheI
HindIII

FIG. 11

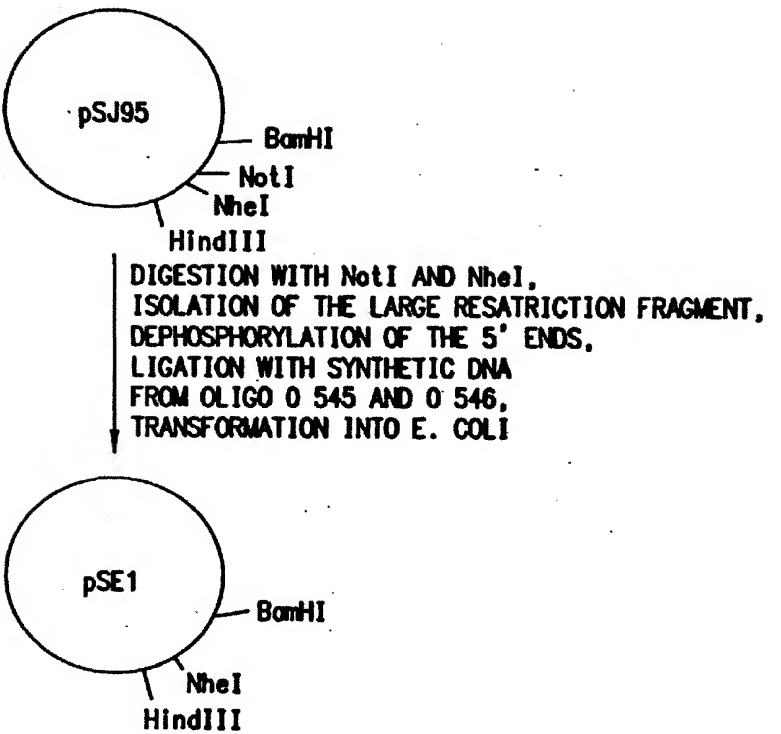
pSJ95
BamHI
NotI
NheI
HindIII
DIGESTION WITH NotI AND NheI,
ISOLATION OF THE LARGE RESATRICTION FRAGMENT,
DEPHOSPHORYLATION OF THE 5' ENDS,
LIGATION WITH SYNTHETIC DNA
FROM OLIGO 0 545 AND 0 546,
TRANSFORMATION INTO E. COLI
pSE1
BamHI
NheI
HindIII

FIG. 12

Figure 13: Amino acid sequence of M37

Met-Ser-Lys-Thr-Cys-Tyr-Glu-Gly-Asn-Gly-His-Phe-Tyr-Arg-Gly-Lys-Ala-Ser-Thr-Asp-Thr-Met-Gly-Arg-Pro-Cys-Leu-Pro-Trp-Asn-Ser-Ala-Thr-Val-Leu-Gln-Gln-Thr-Tyr-His-Ala-His-Arg-Ser-Asp-Ala-Leu-Gln-Leu-Gly-Leu-Gly-Lys-His-Asn-Tyr-Cys-Arg-Asn-Pro-Asp-Asn-Arg-Arg-Arg-Pro-Trp-Cys-Tyr-Val-Gln-Val-Gly-Leu-Lys-Pro-Leu-Val-Gln-Glu-Cys-Met-Val-His-Asp-Cys-Ala-Asp-Gly-Lys-Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu-Leu-Lys-Phe-Gln-Cys-Gly-Gln-Lys-Thr-Leu-Arg-Pro-Arg-Phe-Lys-Ile-Ile-Gly-Gly-Glu-Phe-Thr-Thr-Ile-Glu-Asn-Gln-Pro-Trp-Phe-Ala-Ala-Ile-Tyr-Arg-Arg-His-Arg-Gly-Gly-Ser-Val-Thr-Tyr-Val-Cys-Gly-Gly-Ser-Leu-Ile-Ser-Pro-Cys-Trp-Val-Ile-Ser-Ala-Thr-His-Cys-Phe-Ile-Asp-Tyr-Pro-Lys-Lys-Glu-Asp-Tyr-Ile-Val-Tyr-Leu-Gly-Arg-Ser-Arg-Leu-Asn-Ser-Asn-Thr-Gln-Gly-Glu-Met-Lys-Phe-Glu-Val-Glu-Asn-Leu-Ile-Leu-His-Lys-Asp-Tyr-Ser-Ala-Asp-Thr-Leu-Ala-His-His-Asn-Asp-Ile-Ala-Leu-Leu-Lys-Ile-Arg-Ser-Lys-Glu-Gly-Arg-Cys-Ala-Gln-Pro-Ser-Arg-Thr-Ile-Gln-Thr-Ile-Cys-Leu-Pro-Ser-Met-Tyr-Asn-Asp-Pro-Gln-Phe-Gly-Thr-Ser-Cys-Glu-Ile-Thr-Gly-Phe-Gly-Lys-Glu-Asn-Ser-Thr-Asp-Tyr-Leu-Tyr-Pro-Glu-Gln-Leu-Lys-Met-Thr-Val-Val-Lys-Leu-Ile-Ser-His-Arg-Glu-Cys-Gln-Gln-Pro-His-Tyr-Tyr-Gly-Ser-Glu-Val-Thr-Thr-Lys-Met-Leu-Cys-Ala-Ala-Asp-Pro-Gln-Trp-Lys-Thr-Asp-Ser-Cys-Gln-Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-Cys-Ser-Leu-Gln-Gly-Arg-Met-Thr-Leu-Thr-Gly-Ile-Val-Ser-Trp-Gly-Arg-Gly-Cys-Ala-Leu-Lys-Asp-Lys-Pro-Gly-Val-Tyr-Thr-Arg-Val-Ser-His-Phe-Leu-Pro-Trp-Ile-Arg-Ser-His-Thr-Lys-Glu-Glu-Asn-Gly-Leu-Ala-Leu-Ser-Pro-Val-Val-Ala-Phe-Pro-Arg-Pro-Phe-Leu-Leu-Arg-Asn-Pro-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (SEQ ID NO:24)

Figure 14: Amino acid sequence of M38

Met-Ser-Lys-Thr-Cys-Tyr-Glu-Gly-Asn-Gly-His-Phe-Tyr-Arg-
Gly-Lys-Ala-Ser-Thr-Asp-Thr-Met-Gly-Arg-Pro-Cys-Leu-Pro-
Trp-Asn-Ser-Ala-Thr-Val-Leu-Gln-Gln-Thr-Tyr-His-Ala-His-
Arg-Ser-Asp-Ala-Leu-Gln-Leu-Gly-Leu-Gly-Lys-His-Asn-Tyr-
Cys-Arg-Asn-Pro-Asp-Asn-Arg-Arg-Arg-Pro-Trp-Cys-Tyr-Val-
Gln-Val-Gly-Leu-Lys-Pro-Leu-Val-Gln-Glu-Cys-Met-Val-His-
Asp-Cys-Ala-Asp-Gly-Lys-Lys-Pro-Ser-Ser-Pro-Pro-Glu-Glu-
Leu-Lys-Phe-Gln-Cys-Gly-Gln-Lys-Thr-Leu-Arg-Pro-Arg-Phe-
Lys-Ile-Ile-Gly-Gly-Glu-Phe-Thr-Thr-Ile-Glu-Asn-Gln-Pro-
Trp-Phe-Ala-Ala-Ile-Tyr-Arg-Arg-His-Arg-Gly-Gly-Ser-Val-
Thr-Tyr-Val-Cys-Gly-Gly-Ser-Leu-Ile-Ser-Pro-Cys-Trp-Val-
Ile-Ser-Ala-Thr-His-Cys-Phe-Ile-Asp-Tyr-Pro-Lys-Lys-Glu-
Asp-Tyr-Ile-Val-Tyr-Leu-Gly-Arg-Ser-Arg-Leu-Asn-Ser-Asn-
Thr-Gln-Gly-Glu-Met-Lys-Phe-Glu-Val-Glu-Asn-Leu-Ile-Leu-
His-Lys-Asp-Tyr-Ser-Ala-Asp-Thr-Leu-Ala-His-His-Asn-Asp-
Ile-Ala-Leu-Leu-Lys-Ile-Arg-Ser-Lys-Glu-Gly-Arg-Cys-Ala-
Gln-Pro-Ser-Arg-Thr-Ile-Gln-Thr-Ile-Cys-Leu-Pro-Ser-Met-
Tyr-Asn-Asp-Pro-Gln-Phe-Gly-Thr-Ser-Cys-Glu-Ile-Thr-Gly-
Phe-Gly-Lys-Glu-Asn-Ser-Thr-Asp-Tyr-Leu-Tyr-Pro-Glu-Gln-
Leu-Lys-Met-Thr-Val-Val-Lys-Leu-Ile-Ser-His-Arg-Glu-Cys-
Gln-Gln-Pro-His-Tyr-Tyr-Gly-Ser-Glu-Val-Thr-Thr-Lys-Met-
Leu-Cys-Ala-Ala-Asp-Pro-Gln-Trp-Lys-Thr-Asp-Ser-Cys-Gln-
Gly-Asp-Ser-Gly-Gly-Pro-Leu-Val-Cys-Ser-Leu-Gln-Gly-Arg-
Met-Thr-Leu-Thr-Gly-Ile-Val-Ser-Trp-Gly-Arg-Gly-Cys-Ala-
Leu-Lys-Asp-Lys-Pro-Gly-Val-Tyr-Thr-Arg-Val-Ser-His-Phe-
Leu-Pro-Trp-Ile-Arg-Ser-His-Thr-Lys-Glu-Glu-Asn-Gly-Leu-
Ala-Leu-Ser-Pro-Val-Val-Ala-Phe-Pro-Arg-Pro-Phe-Leu-Leu-
Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-Glu-Glu-Tyr-Leu-
Gln (SEQ ID NO:25)

CHIMERIC PROTEINS HAVING FIBRINOLYTIC AND THROMBIN-INHIBITING PROPERTIES

This application is a continuation of application Ser. No. 08/563,649 filed on Nov. 28, 1995.

BACKGROUND OF THE INVENTION

This invention relates to chimeric proteins having fibrinolytic and thrombin-inhibiting properties, which are linked at the C-terminal end of the plasminogen-activating amino acid sequence to a thrombin-inhibiting amino acid sequence. The invention also relates to plasmids for producing these polypeptides and to thrombolytic agents which contain a polypeptide of this type as their active ingredient.

In all industrialized countries, cardio-circulatory diseases currently constitute the most frequent cause of death. Particularly important in this respect are acute thrombotic occlusions, the occurrence of which in the case of coronary thrombosis leads within a very short time to a life-threatening under-supply of the cardiac muscle. Similar considerations apply to cerebral thrombosis, intracerebral occlusions being accompanied here by massive ischemic damage to the brain areas concerned. In contrast to coronary thrombosis, which is associated with high mortality rates, under-supply in cerebral thrombosis does not as a rule lead to life-threatening situations, but to severe impairment of an everyday way of life due to the failure of certain brain functions, and thus leads in part to a drastic loss of quality of life for those affected. It is generally true for both these forms of thrombosis that within a few hours—without therapy—the regions supplied by the arteries concerned are irreversibly damaged. Other thrombotic occlusion diseases which require treatment include pulmonary embolism, venous thrombosis and peripheral arterial occlusion diseases.

The occlusion of a blood vessel caused by a thrombus mainly occurs at an arteriosclerotic lesion comprising fibrin, thrombocytes and erythrocytes under the action of various enzymes of the blood coagulation system. Within the enzyme cascade of the coagulation system, thrombin plays a prominent role. Thrombin can activate all the important enzymes of the coagulation system, can induce the aggregation of thrombocytes and can lead to the formation of a fibrin network by the conversion of fibrinogen to fibrin (Furie and Furie in New Engl. J. Med. 326, 800 (1992)).

The formation of thromboses is restricted by physiological anticoagulants, for example antithrombin III, activated protein C and tissue factor pathway inhibitor. Once formed, thromboses can be re-dissolved by the action of plasmin occurring naturally in the body. Plasmin is formed from an inactive proenzyme, plasminogen, which is proteolytically activated by plasminogen activators. The thrombolysis due to plasmin is utilized therapeutically, by treating patients with thrombotic diseases, particularly patients with acute coronary thrombosis, with plasminogen activators. The aim of therapeutic intervention is to reduce the infarct and to lower the mortality rate. Streptokinase, APSAC (anisolated plasminogen streptokinase activator complex), double-chain urokinase (UK), recombinant single-chain urokinase (recombinant prourokinase) and tissue plasminogen activator (t-PA) are currently available for this therapy (Collen and Lijnen in Blood 78, 3114, (1991)). It clearly follows from the experiences of lysis therapy which have been published hitherto that re-opening of the occluded coronary vessels within a few hours, i.e. 1 to 4 hours after the occurrence of the coronary, provides the best functional results. In order to achieve the aim of optimum reperfusion, therapy in the majority of cases should actually be commenced even before admission as an in-patient. However, this is only possible using a fibrinolytic agent which has few side effects and which is safe, and in view of the diagnosis situation also, which is still uncertain at this time. When employed in the requisite doses for the treatment of acute coronary disease, however, all fibrinolytic agents of the so-called first generation, such as streptokinase, APSAC and urokinase, produce a generalized plasminogen activation which is accompanied by a high risk of hemorrhage. Even the use of fibrinolytic agents of the so-called second generation, t-PA and prourokinase, leads to systemic plasminogen activation in many coronary patients. For successful reperfusion and to prevent re-occlusions, both t-PA and prourokinase have to be used in high doses, which result in significant fibrinogenolysis, and therefore to systemic plasminogen activation. This is in agreement with the observation that in previous studies no significant differences could be detected in the frequency of hemorrhage complications between patients treated with tPA or prourokinase and patients treated with streptokinase.

Various approaches have therefore been pursued aimed at improving the pharmacological profile of plasminogen activators. The following are under development: bat plasminogen activators (Gardell et al. in J. Biol. Chem. 264, 17947 (1989); Australian Patent No. AU 642,404-B (=EP 383,417), staphylokinase (Schlott et al. in Bio/Technology 12, 185 (1994); Collen and Van De Werf in Circulation 87, 1850 (1993)), the recombinant tissue plasminogen activator BM 06.022 (Martin et al. in J. Cardiovasc. Pharm. 18, 111 (1991)) and the t-PA variant TNK-t-PA (Keyt et al. in Proc. Natl. Acad. Sci. 91, 3670 (1994)).

Streptokinase, a protein of hemolytic Streptococci, activates human plasminogen, in that it forms a complex with plasminogen and thereby converts the plasminogen into an active conformation. This complex itself converts free plasminogen to plasmin, which then in turn cleaves the plasminogen bound to streptokinase. Staphylokinase, a protein obtained from *Staphylococcus aureus*, also acts similarly, but possesses a higher fibrin specificity compared with streptokinase. APSAC, a compound of streptokinase and human plasminogen which is produced in vitro, is a further development of streptokinase. Due to a chemical modification of the active center of the plasminogen, APSAC has a biological half-life which is longer than that of streptokinase.

Urokinase is a human protein which can be obtained in two forms as a proteolytically active protein from urine; high molecular weight urokinase (HUK) and low molecular weight urokinase (LUK) (Stump et al. in J. Biol. Chem. 261, 1267 (1986)). HUK and LUK are active forms of urokinase, i.e. double-chain molecules. Urokinase is formed as single-chain urokinase (prourokinase) in various tissues and can be detected in small amounts as a proenzyme in human blood (Wun et al. in J. Biol. Chem. 257, 3276 (1982)). As HUK, the activated form of prourokinase has a molecular weight of 54 kilodaltons and consists of 3 domains: the amino-terminal growth factor domain, the kringle domain and the serine protease domain (Guenzler et al. in Hoppe-Seyler's Z. Physiol. Chem. 363, 1155 (1982); Steffens et al. in Hoppe-Seyler's Z. Physiol. Chem. 363, 1043 (1982)). Although prourokinase and plasminogen are present as proenzymes, prourokinase is capable, due to its intrinsic activity, of transforming plasminogen into active plasmin. However, this plasminogen activator does not attain its full activity

until the plasmin formed has itself cleaved the prourokinase between ^{158}lysine and ^{159}isoleucine (Lijnen et al. in J. Biol. Chem. 261, 1253 (1986)). The production of urokinase in *Escherichia coli* by genetic engineering was first described by Heyneker et al. (Proceedings of the IVth International Symposium on Genetics of Industrial Microorganisms 1982). Unglycosylated prourokinase (saruplase) is produced using a synthetic gene (Brigelius-Flohe' et al. in Appl. Microbiol. Biotech. 36, 640 (1992)).

t-PA is a protein with a molecular weight of 72 kilodaltons which is present in blood and in tissue. This plasminogen activator consists of 5 domains: the amino-terminal finger domain, the growth factor domain, kringle domain 1, kringle domain 2 and the serine protease domain. Like prourokinase, t-PA is converted into the active, double-chain form by a plasmin-catalyzed cleavage between kringle domain 2 and the serine protease domain, i.e. between ^{275}Arg and ^{276}Ile. In vitro studies and the results of experiments on animals indicate that t-PA binds to fibrin and its enzymatic activity is stimulated by fibrin (Collen and Lijnen in Blood 78, 3114 (1991)). The fibrin specificity of t-PA should prevent the formation of plasmin in the entire blood system, resulting not only in the decomposition of fibrin decomposed but also in the decomposition of fibrinogen. A systemic plasminogen activation such as this as well as the extensive decomposition of fibrinogen are undesirable, since this increases the risk of hemorrhage. It has been shown in therapeutic practice, however, that the expectations derived from pre-clinical studies as regards the fibrin specificity of t-PA are not fulfilled. Due to the short biological half-life of t-PA it is necessary to infuse high doses, which result in systemic plasminogen activation despite this fibrin specificity (Keyt et al. in Proc. Natl. Acad. Sci. 91, 3670 (1994)).

r-PA and TNK-t-PA are variants of t-PA which possess improved properties. In r-PA (BM 06.022) the first three t-PA domains, i.e. the finger domain, the growth factor domain and the first kringle domain, have been deleted, so that the shortened molecule only contains the second kringle domain and the protease domain. r-PA is produced in *Escherichia coli* by genetic engineering and is not glycosylated. Compared with t-PA, r-PA has a longer biological half-life and more rapidly leads to reperfusion. It has been shown in experiments on animals that r-PA applied as a bolus is just as effective as a t-PA infusion (Martin et al. in J. Cardiovasc. Pharmacol. 18, 111 (1991)).

The t-PA variant TNK-t-PA differs from natural t-PA on three counts: the replacement of ^{103}threonine by asparagine, due to which a new glycosylation site is formed; the replacement of ^{117}asparagine by glutamine, due to which a glycosylation site is removed, and the replacement of the sequence between ^{296}lysine and ^{299}arginine by four successive alanine units. The combination of these three mutations results in a polypeptide with a higher fibrin specificity and a longer biological half-life compared with natural t-PA. Moreover, TNK-t-PA is considerably less inhibited by PAI-1 than is natural t-PA (Keyt et al. in Proc. Natl. Acad. Sci. 91, 3670 (1994)). Results obtained from experiments on animals in which a precursor of TNK-t-PA was used indicate that TNK-t-PA is suitable for bolus application (Refino et al. in Thromb. Haemost. 70, 313 (1993)).

Bat plasminogen activator (bat-PA) occurs in the saliva of the *Desmodus rotundus* bat. This plasminogen activator, which has meanwhile also been synthesized by genetic engineering, has an even more pronounced fibrin specificity than t-PA and in tests on animals has exhibited improved thrombolysis with an increased biological half-life and reduced systemic plasminogen activation (Gardell et al. in Circulation 84, 244 (1991)).

In the treatment of thrombotic diseases, plasminogen activators are generally administered together with an anticoagulant substance, for example heparin. This results in improved thrombolysis compared to treatment with only a plasminogen activator (Tebbe et al. in Z. Kardiol. 80, Suppl. 3, 32 (1991)). Various clinical results indicate that, in parallel with the dissolution of thromboses, an increased tendency towards coagulation occurs (Szczeklik et al. in Arterioscl. Thromb. 12, 548 (1992); Goto et al. in Angiology 45, 273 (1994)). It is assumed that thrombin molecules which are enclosed in the thrombus and which are released again when the clot dissolves are responsible for this. Moreover, there are indications that plasminogen activators themselves also accelerate the activation of prothrombin and thus act in opposition to thrombolysis (Brommer and Meijer in Thromb. Haemostas. 70, 995 (1993)). Anticoagulant substances such as heparin, hirugen, hirudin, argatroban, protein C and recombinant tick anticoagulant peptide (TAP) can suppress this increased tendency towards re-occlusion during thrombolysis and can thus enhance the success of lysis therapy (Yao et al. in Am. J. Physiol. 262 (Heart Circ. Physiol. 31) H 347–H 379 (1992); Schneider in Thromb. Res. 64, 667 (1991); Gruber et al. in Circulation 84, 2454 (1991); Martin et al. in J. Am. Coll. Cardiol. 22, 914 (1993); Vlasuk et al. in Circulation 84, Suppl. II-467 (1991).

One of the strongest thrombin inhibitors is hirudin from the *Hirudo medicinales* leech, which consists of 65 amino acids. There are various iso-forms of hirudin, which differ as regards some of their amino acids. All iso-forms of hirudin block the binding of thrombin to a substrate, for example fibrinogen, and also block the active center of thrombin (Rydel et al. in Science 249, 277 (1990); Bode and Huber in Molecular Aspects of Inflammation, Springer, Berlin, Heidelberg, 103–115 (1991); Stone and Hofsteenge in Prot. Engineering 2, 295 (1991); Dodt et al. in Biol. Chem. Hoppe-Seyler 366, 379 (1985). In addition, smaller molecules derived from hirudin are known, which also act as thrombin inhibitors (Maraganore et al. in Biochemistry 29, 7095 (1990); Krstenansky et al. in J. Med. Chem. 30, 1688 (1987); Yue et al. in Prot. Engineering 5, 77 (1992)).

The use of hirudin in combination with a plasminogen activator for the treatment of thrombotic diseases is described in U.S. Pat. No. 4,944,943 (=EP 328,957) and U.S. Pat. No. 5,126,134 (=EP 365,468). The use of hirudin derivatives in combination with a thrombolytic agent is known from PCT International Patent Application WO 91/01142.

Hirullin is a protein containing 61 amino acids which is isolated from the *Hirudo manillensis* leech. Hirullin is identical to hirudin as regards its action and inhibitor strength, but differs very considerably from hirudin as regards its amino acid sequence. It has also proved possible to derive smaller molecules from hirullin, which are very good thrombin inhibitors (Krstenansky et al. in Febs Lett. 269, 465 (1990)).

In addition, thrombin can also be inhibited by a peptide which is derived from the amino-terminal sequence of the human thrombin receptor (Vu et al. in Nature 253, 674 (1991)). The thrombin receptor contains a thrombin-binding sequence, with an adjacent cleavage site for thrombin, in the extracellular, amino-terminal region. This sequence can inhibit thrombin provided that the cleavage site is masked by the replacement of ^{42}serine by ^{42}phenylalanine.

Phaneuf et al., in Thromb. Haemost. 71, 481 (1994), describe a complex which results from a fortuitous chemical linking of streptokinase and hirudin. The plasminogen-

activating capacity of this streptokinase-hirudin complex is less than that of unmodified streptokinase by a factor of 8, however.

As noted above, plasminogen-activating amino acid sequences contain various domain sites which are well known and are described in the literature.

Urokinase and prourokinase comprise the following domains:

Domain	Amino Acids Included
Growth Factor Domain	amino acids 1 to 43
Kringle Domain	amino acids 50 to 131
Serine Protease Domain	amino acids 158 to 411

See Guenzler et al., "The Primary Structure of High Molecular Mass Urokinase form Human Urine; The Complete Amino Acid Sequence of the A Chain", *Hoppe-Seyler's Z. Physiol. Chem.*, 363, 1:55–65 (1982); Steffens et al., "The Complete Amino Acid Sequence of Low Molecular Mass Urokinase from Human Urine", *Hoppe-Seyler's Z. Physiol. Chem.*, 363, 1043–1058 (1982).

Tissue plasminogen activator comprises the following domains:

Domain	Amino Acids Included
Finger Domain	amino acids 4 to 50
Growth Factor Domain	amino acids 50 to 87
Kringle 1 Domain	amino acids 87 to 176
Kringle 2 Domain	amino acids 176 to 262
Serine Protease Domain	amino acids 276 to 527

See Collen et al., "Thrombolytic and Pharmacokinetic Properties of Human Tissue-Type Plasminogen Activator Variants Obtained by Deletion and/or Duplication of Structural/Functional Domains, in a Hamster Pulmonary Embolism Model", *Thrombosis and Haeomostasis*, 65, (2), 174–180 (1991).

Bat-plasminogen activator comprises the following domains:

Domain	Amino Acids Included
Finger Domain	amino acids 1 to 43
Growth Factor Domain	amino acids 44 to 84
Kringle Domain	amino acids 92 to 173
Serine Protease Domain	amino acids 189 to 441

See Gardell et al., "Isolation, Characterization, and cDNA Cloning of a Vampire Bat Salivary Plasminogen Activator", *Journal of Biological Chem-st-y*, 264, (30), 17947–952 (1989).

SUMMARY OF THE INVENTION

The underlying object of the present invention was to provide active ingredients for the treatment of vascular diseases caused by thrombosis, Which effect complete thrombolysis within a very short period and which at the same time prevent vascular re-occlusion after what is first of all a successful thrombolysis.

Another object of the invention was to provide a way to prevent systemic plasminogen activation by means of these active ingredients.

In accordance with the present invention it has now been found that the considerable demands imposed on such active ingredients can be fulfilled by chimeric proteins having fibrinolytic properties which contain a thrombin-inhibiting amino acid. sequence at the C-terminal end of the plasminogen-activating amino acid sequence.

Accordingly, the present invention relates to chimeric proteins having fibrinolytic and thrombin-inhibiting properties, which are linked at the C-terminal end of the plasminogen-activating amino acid sequence to an amino acid sequence of formula I

Ser-X_1-X_2-X_3-X_4-X_5-Pro-Arg-Pro-Y_1-Y_2-Y_3-Y_4-Asn-Pro-Z

(SEQ ID NO: 1),
in which X_1 represents Pro or Leu; X_2 represents Gly, Val or Pro; X_3 represents Lys, Val, Arg, Gly or Glu; X_4 represents Ala, Val, Gly, Leu or Ile; X_5 represents Gly, Phe, Trp, Tyr or Val; Y_1 represents Phe, Tyr or Trp; Y_2 represents Leu, Ala, Gly, Ile, Ser or Met; Y_3 represents Leu, Ala, Gly, Ile, Ser or Met; Y_4 represents Arg, Lys or His, and Z represents the amino acid sequence of formula II

Gly-Asp-Z_1-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln

(SEQ ID NO: 2),
in which Z_1 represents Phe or Tyr,
or of formula III

Asn-Asp-Lys-Tyr-Glu-Pro-Phe-Glu-Glu-Tyr-Leu-Gln

(SEQ ID NO: 3),
or of formula IV

Ser-Asp-Phe-Glu-Glu-Phe-Ser-Leu-Asp-Asp-Ile-Glu-Gln

(SEQ ID NO: 4),
or of formula V

Ser-Glu-Phe-Glu-Glu-Phe-Glu-Ile-Asp-Glu-Glu-Glu-Lys

(SEQ ID NO: 5).

The chimeric proteins according to the invention bind to thrombin via the thrombin-inhibiting amino acid sequence of formula I, due to which high concentrations of chimeric protein are attained at the clot. Since the clots formed in acute coronary or cerebral thrombosis are rich in thrombin, the thrombus specificity of the proteins according to the invention provides the possibility of increasing the thrombolytic efficacy and selectivity of the plasminogen activators. Systemic plasminogen activation and fibrinogenolysis are thereby prevented and the level of safety of the active ingredients is considerably enhanced. Due to the thrombus specificity, the dose can also be reduced compared with conventional plasminogen activators, which again enhances the safety of the preparation. At the same time it can be anticipated that the dosage of the anticoagulant co-medication (e.g. containing heparin) can be reduced when using the proteins according to the invention. Further, it is also possible to dispense with an additional anticoagulant.

Preferred chimeric proteins contain as their plasminogen-activating amino acid sequence the unaltered amino acid sequence of prourokinase, at least one modified prourokinase amino acid sequence having a serine protease domain exhibiting at least 90%, and preferably at least 95%, sequence identity to the serine protease domain of the unaltered amino acid sequence of prourokinase, the unaltered amino acid sequence of urokinase, at least one modi-

fied urokinase amino acid sequence having a serine protease domain exhibiting at least 90%, and preferably at least 95%, sequence identity to the serine protease domain of the unaltered amino acid sequence of urokinase, the unaltered amino acid sequence of tissue plasminogen activator (t-PA), at least one modified t-PA amino acid sequence having a serine protease domain exhibiting at least 90%, and preferably at least 95%, sequence identity to the serine protease domain of the unaltered amino acid sequence of t-PA, the unaltered amino acid sequence of bat plasminogen activator (bat-PA), at least one modified bat-PA amino acid sequence having a serine protease domain exhibiting at least 90%, and preferably at least 95%, sequence identity to the serine protease domain of the unaltered amino acid sequence of bat-PA, and/or the amino acid sequence of streptokinase, staphylokinase and/or APSAC. As used herein, the term "modified" amino acid sequence refers to an amino acid sequence which has been altered by deletion, substitution, insertion and/or addition. Such deletions, substitutions, insertions and/or additions may be effected by conventional techniques which are known to persons skilled in the genetic engineering art.

In particular, the plasminogen-activating amino acid sequence in the proteins according to the invention contains the unaltered amino acid sequence of prourokinase, at least one modified prourokinase amino acid sequence having a serine protease domain exhibiting at least 90%, and preferably at least 95%, sequence identity to the serine protease domain of the unaltered amino acid sequence of prourokinase, the unaltered amino acid sequence of t-PA and/or at least one modified t-PA amino acid sequence having a serine protease domain exhibiting at least 90%, and preferably at least 95%, sequence identity to the serine protease domain of the unaltered amino acid sequence of t-PA.

In preferred embodiments of the invention, the plasminogen-activating amino acid sequence comprises a modified sequence which includes at least one kringle domain exhibiting at least 90% sequence identity to the corresponding kringle domain of the corresponding unaltered sequence, and a serine protease domain exhibiting at least 90% sequence identity to the corresponding serine protease domain of the corresponding unaltered sequence.

Proteins are particularly preferred -n which the plasminogen-activating amino acid sequence comprises at least one of the sequence of prourokinase which consists of 411 amino acids and in which the amino acid in position 407 is Asn or Gln, or the ^{47}Ser to ^{411}Leu amino acid sequence of prourokinase in which the amino acid in position 407 is Asn or Gln; or the ^{136}Ser to ^{411}Leu amino acid sequence of prourokinase in which the amino acid in position 407 is Asn or Gln; or the unaltered sequence of t-PA which consists of 527 amino acids; or the Ser-^{89}Arg to ^{527}Pro amino acid sequence of t-PA, or the ^{174}Ser to ^{527}Pro amino acid sequence of t-PA.

In the chimeric proteins, the plasminogen-activating amino acid sequence at the C-terminal end is preferably linked to a thrombin-inhibiting amino acid sequence of formula I, in which X_1 represents Pro; X_2 represents Val; X_3 represents Lys or Val; X_4 represents Ala, and X_5 represents Phe. In the amino acid sequence of formula I, Y_1 preferably represents Phe; Y_2 preferably represents Leu; Y_3 preferably represents Leu, and Y_4 preferably represents Arg. In particular, the variable Z in the amino acid sequence of formula I represents an amino acid sequence of formula II or formula IV.

Compared with known plasminogen activators, or with known mixtures comprising a plasminogen activator and a thrombin inhibitor, or with the known streptokinase-hirudin complex, the proteins according to the invention are distinguished by a stronger fibrinolytic effect combined with surprisingly good thrombin-inhibiting properties. In addition, plasma fibrinogen is consumed in considerably smaller amounts by the polypeptides according to the invention. The effect of the significantly higher fibrin specificity which results from this, particularly by comparison even with the known mixtures comprising a plasminogen activator and a thrombin inhibitor, is that the coagulation capacity of the blood is only slightly affected and the risk of uncontrolled hemorrhages as possible complications of systemic fibrinogen decomposition is minimized. The high fibrin specificity of the proteins according to the invention thus permits bolus applications with a significantly reduced risk of hemorrhage compared with bolus applications of known thrombolytic agents.

Accordingly, the present invention also relates to thrombolytic agents which contain a protein according to the invention as their active ingredient.

From 0.1 to 1 mg of a polypeptide according to the invention is required per kg for the treatment of vascular occlusions caused by thrombosis, for example coronary thrombosis, cerebral thrombosis, peripheral acute arterial occlusion, pulmonary embolism, unstable angina pectoris and deep venous thrombosis of the legs and pelvis. The proteins according to the invention can be administered parenterally by bolus injection or infusion.

In addition to at least one polypeptide according to the invention, the thrombolytic agents according to the invention may contain auxiliary materials or adjuvants, for example carriers, solvents, diluents, colorants and binders. The choice of these auxiliary materials, as well as the amounts thereof to be used, depends on how the drug is to be administered, and is considered to be within the skill of the art.

The proteins according to the invention are produced using genetic engineering methods. For this purpose the corresponding genes from synthetic oligonucleotides are cloned into suitable plasmids and expressed in *Escherichia coli* under the control of the trp- or tac promoter, particularly under the control of the trp promoter.

Accordingly, the present invention also relates to plasmids for use in the production of chimeric proteins which plasmids comprise operons which comprise a regulable promoter, a Shine-Dalgarno sequence which is effective as a ribosome binding site, a start codon, a synthetic structural gene for a protein according to the invention, and one or two terminators downstream of the structural gene.

The plasmids according to the invention can be expressed in *Escherichia coli* strains, particularly in *Escherichia coli* strains of group K 12, for example *E. coli* K 12 JM 101 (ATCC 33876), *E. coli* K 12 JM 103 (ATCC 39403), *E. coli* K 12 JM 105 (DSM 4162) and *E. coli* K 12 DH 1 (ATCC 33849). In the bacterial cell, the polypeptides according to the invention occur in high yield in inclusion bodies in which the protein exists in denatured form. After isolating the inclusion bodies the denatured protein is folded into the desired tertiary structure, by a protein chemistry technique, under the action of a redox system.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be described in further detail hereinafter with reference to representative examples illustrated in the accompanying drawings in which:

FIGS. 1 through 12 are schematic illustrations of the steps for preparing expression plasmids for producing the proteins of the present invention;

FIG. 13 shows the amino acid sequence of peptide M37 (SEQ ID NO: 24); and

FIG. 14 shows the amino acid sequence of peptide M38 (SEQ ID NO: 25).

EXAMPLES

1. Preparation, isolation and purification of proteins according to the invention.

a) Cloning operations

The expression plasmids for the production by genetic engineering of the polypeptides according to the invention in *Escherichia coli* were prepared in a manner known in the art. The sequence of the individual preparation steps is illustrated in FIGS. 1 to 12. The starting materials for the preparation of the plasmids were the plasmids pBluescript KS II+(manufactured by Stratagene, Heidelberg), pUC8 (manufactured by Pharmacia, Freiburg), and pGR201. pGR201 is identical to plasmid pSF160 described in Canadian Patent Application No. CA 2,020,656 (=EP 408, 945) and Appl. Microbiol. Biotechn. 36, 640–649 (1992). The restriction endonucleases BanII, BamHI, ClaI, HindIII, NcoI, NdeI, NheI, NotI, and the DNA-modifying enzymes such as the alkaline phosphatase, T4 ligase, T4 kinase and T7 polymerase, were obtained from the companies Pharmacia, Stratagene, Boehringer Mannheim and Gibco (Eggenstein). The changes in the plasmids during their preparation were verified by restriction analysis and DNA sequencing. DNA sequencing was effected according to the manufacturer's instructions, using a collection of reagents supplied by Pharmacia. Various oligodeoxyribonucleotides (oligos) were used in the preparation of the plasmids; their sequences, together with the associated designations, are given in Table 1.

The oligodeoxyribonucleotides were prepared in detritylated form on an 0.1 μmolar scale, by means of a synthesizer (Model 391) supplied by Applied Biosystems (Weiterstadt) according to the manufacturer's data, using β-cyanoethyl-protected diisopropylamino-phosphoamidites. 100 pmoles of each oligodeoxyribonucleotide were phosphorylated with one T4 kinase enzyme unit in the presence of 10 mM adenosine triphosphate in 50 mM tri(hydroxymethyl)aminomethane/HCl (tris-HCl), 10 mM magnesium chloride and 5 mM dithiothreitol at a pH of 7.5 and subsequently transformed to double-strand DNA molecules in the same buffer. The resulting synthetic double-strand DNA molecules were purified by gel electrophoresis on a polyacrylamide gel (5% polyacrylamide) and subsequently used in the ligation with the correspondingly prepared plasmids. Preparation of the plasmids by digestion with restriction enzymes, isolation of the corresponding restriction fragments and dephosphorylation of the 5'-ends, subsequent ligation and transformation into *E. coli* K12 JM103, as well as all other genetic engineering operations, were carried out in a known manner as described by Sambrook et al. in "Molecular Cloning: A Laboratory Manual", Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, USA, 1989.

TABLE 1

Oligo Sequence written from 5' to 3'
O 105 TATGAGCAAAACTTGCTACGAAGGTAACGGTCACTTCTACCGTG GTAAGGCTTCTACCGACAC (SEQ ID NO:6)
O 106 CATGGTGTCGGTAGAAGCCTTACCACGGTAGAAGTGACCGTTAC CTTCGTAGCAAGTTTTGCTCA (SEQ ID NO:7)

TABLE 1-continued

Oligo Sequence written from 5' to 3'
O 220 CGGTTAAGGCTTTCCCGAGGCCTGGTGGTGGTGGTAACGGTGAC TTCGAAGAAATCCCGGAAGAGTACCTGTGATAGGATCAA (SEQ ID NO:8)
O 221 CTAGTTGATCCTATCACAGGTACTCTTCCGGGATTTCTTCGAAG TCACCGTTACCACCACCACCAGGCCTCGGGAAAGCCTTAACCGG GCT (SEQ ID NO:9)
O 265 CACCCGGCGGAGACGGCGGCTCAGAGCCAGACCGTTTTCTTCT TTGGTGTGAGAACG (SEQ ID NO:10)
O 281 CGTCCGGGTGGTGGTGGTAACGGTGACTTCGAAGAAATCCCGGA AGAATACCTGTAAG (SEQ ID NO:11)
O 282 GATCCGTTCTCACACCAAAGAAGAAAACGGTCTGGCTCTGAGCC CGCCGTCTCCGCCGGGTGGTTTCCCG (SEQ ID NO:12)
O 283 CTAGCTTACAGGTATTCTTCCGGGATTTCTTCGAAGTCACCGTT ACCACCACCACCGGACGCGGGAAAC (SEQ ID NO:13)
O 329 AAGAAATCCCGGAAGAATACCTGCAATAAG (SEQ ID NO:14)
O 330 CGGTTAAGGCTTGGGGACCGCGGCCGCTGGGTGGTGGTGGTAAC GGTGACTTCG (SEQ ID NO:15)
O 331 ACCACCACCCAGCGGCCGCGGTCCCCAAGCCTTAACCGGGCT (SEQ ID NO:16)
O 332 CTAGCTTATTGCAGGTATTCTTCCGGGATTTCTTCGAAGTCACC GTTACC (SEQ ID NO:17)
O 347 CGGTTGTTGCTTTCCCGC (SEQ ID NO:18)
O 348 GGCCGCGGGAAAGCAACAACCGGGCT (SEQ ID NO:19)
O 545 CTAGCTTATTGCAGGTATTCTTCGAACGGTTCGTATTTGTCGTT AGGGTTACGCAGCAGGAAA (SEQ ID NO:20)
O 546 GGCCTTTCCTGCTGCGTAACCCTAACGACAAATACGAACCGTTC GAAGAATACCTGCAATAAC (SEQ ID NO:21)
O 615 CTAGCTTATTGCAGGTATTCTTCCGGGATTTCTTCGAAGTCACC AGGGTTACGCAGCAGGAAA (SEQ ID NO:22)
O 618 GGCCTTTCCTGCTGCGTAACCCTGGTGACTTCGAAGAAATCCCG GAAGAATACCTGCAATAAG (SEQ ID NO:23)

b) Preparation of reusable cultures and fermentation

The recombinant expression plasmids pSEI (M 38) and pSE9 (M 37) were introduced into *E. coli* K12 JM103 (ATCC 39403) and spread out on standard I-nutrient agar (Merck, 150 mg/l ampicillin) (Sambrook et al. "Molecular Cloning: A Laboratory Manual"). A single colony of each transformation was cultivated in standard I-nutrient broth (Merck, pH 7.0; 150 mg/l ampicillin) at 20° C. to an optical density (OD) of 1 at 578 nm, and, with the addition of dimethyl sulfoxide (DMSO) (final concentration 7.5%), was frozen at and stored at −70° C. in 2 ml portions as a reusable culture. To produce the polypeptides according to the invention, 1 ml of each reusable culture was suspended in 20 ml standard I-nutrient broth (pH 7.0; 150 mg/l ampicillin) and cultivated at 37° C. to an OD of 1 at 578 nm.

The entire amount of culture obtained was then suspended in 1 liter of standard I-nutrient broth (pH 7.0; 150 mg/l ampicillin) and fermented in shaken flasks at 37° C. Induction was effected by adding 2 ml of indole-acrylacetic acid solution (60 mg in 2 ml ethanol) at an OD of 0.5 to 1 at 578 nm.

c) Expression testing

In order to test the expression rate, cells corresponding to 1 ml of a cell suspension with an OD of 1 at 578 nm were

centrifuged directly before induction and every hour after induction (for a total of 6 hours). The sedimented cells were digested with lysozyme (1 mg lysozyme per ml in 50 mM tris-HCl buffer, pH 8.0, 50 mM ethylenediaminetetraacetic acid (EDTA) and 15% saccharose) The homogenate from the lysed cells was solubilized in 4–5 M guanidinium hydrochloride solution and after diluting to 1.2 M guanidinium hydrochloride and adding a reducing agent (glutathione or cysteine) was subjected to the folding reaction for 2–5 hours (Winkler et al., Biochemistry 25, 4041 to 4045 (1986)). The single-chain polypeptides according to the invention which were obtained were transformed into the corresponding double-chain molecules by the addition of plasmin, and the activity of the double-chain molecules was determined with the chromogen substrate pyro-Glu-Gly-Arg-p-nitroanilide. Activation of the polypeptides according to the invention with plasmin was effected in 50 mM tris-HCl buffer, 12 mM sodium chloride, 0.02% Tween 80 at pH 7.4 and 37° C. The ratio of polypeptide according to the invention to plasmin was about 8000–36,000 to 1, based on enzyme units. The test incubation was effected in 50 mM tris-HCl buffer and 38 mM sodium chloride at pH 8.8 in the presence of 0.36 μM aprotinine (to inhibit the plasmin) and 0.27 mM of pyro-Glu-Gly-Arg-p-nitroanilide substrate at 37° C. Depending on the concentration of the polypeptide according to the invention, the reaction was stopped after an incubation period of 5 to 60 minutes by adding 50% acetic acid, and the extinction at 405 nm was measured. According to the information from the manufacturer of the substrate (Kabi Vitrum, Sweden), in this procedure a change in extinction of 0.05 per minute at 405 nm corresponds to a urokinase activity of 25 ploug units per ml of test solution. The polypeptides according to the invention had specific activities between 120,000 and 155,000 ploug units per mg of protein. The protein content of the solutions was determined using the BCA assay of the Pierce company.

d) Isolation and purification

After 6 hours, the fermentation carried out under the conditions described in 1b) was terminated (density 5–6 CD at 578 nm) and the cells were extracted by centrifuging. The cell sediment was re-suspended in 200 ml water and digested in a high-pressure homogenizer. After renewed centrifugation, the sediment, which contained the entire amount of single-chain polypeptide according to the invention, was dissolved in 500 ml 5 M guanidinium hydrochloride, 40 mM cysteine, 1 mM EDTA at a pH of 8.0 and diluted with 2000 ml 25 mM tris-HCl with a pH of 9.0. The folding reaction was complete after about 12 hours.

After adding 8 g silica gel, the polypeptides according to the invention which were obtained were completely bound to silica gel by stirring for 2 hours. The loaded silica gel was separated and washed with acetate buffer (pH 4.0). The polypeptides were eluted with 0.5 M trimethylammonium chloride (TMAC) in 0.1 M acetate buffer (pH 4). After two chromatographic separations (copper chelate column and cation exchanger) the polypeptides were obtained in pure form. Their single-chain character was established by N-terminal sequence analysis.

The isolated polypeptides according to the invention, the amino acid sequences of which are given in FIGS. 13 and 14, exhibited no activity or only very slight activity (less than 1%) in a direct activity test with the chromogen substrate for urokinase. Full enzyme activity was only obtained after cleavage with plasmin (the conditions are given in Section 1c). The polypeptides according to the invention were accordingly expressed as single-chain proteins in *E. coli* K12 JM103.

2. Determination of the thrombin-inhibiting effect

The inhibitor effect of the polypeptides according to the invention was determined by measuring the thrombin time, by mixing 200 μl of a 1:10 dilution of human citrate plasma in veronal buffer with 50 μl of thrombin solution (0.2 units) and 50 μl of an aqueous solution containing 0.4–30 μg of a polypeptide according to the invention. The time to the formation of a fibrin network was then measured.

The thrombin time values listed in Table 2 were determined in the presence of prourokinase or of the proteins M 37 and M 38 according to the invention. In contrast to prourokinase, M 37 and M 38 prolong the thrombin time depending on their dosage, and thus act as coagulation inhibitors.

TABLE 2

	Thrombin Time [sec]		
Protein [μg]	Prourokinase	M 37	M 38
0	31	32	32
0.4		40	
0.8		79	
1.2		148	
1.6		195	
2.0		266	
4.0	31	>300	58
8.0			81
12.0			104
16.0			130
20.0	33		150
30.0	33		>300

The foregoing description and examples have been set forth merely to illustrate the invention and are not intended to be limiting. Since modifications of the described embodiments incorporating the spirit and substance of the invention may occur to persons skilled in the art, the invention should be construed broadly to include all variations falling within the scope of the appended claims and equivalents thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 25

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids

-continued

```
          (B) TYPE: amino acid
          (C) STRANDEDNESS:
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

      (v) FRAGMENT TYPE: C-terminal

     (ix) FEATURE:
          (A) NAME/KEY: Modified-site
          (B) LOCATION:2..6
          (D) OTHER INFORMATION:/product= "Xaa"
               /label= Xaa
               /note= "Pos 2: Xaa = Pro, Leu
               Pos 3: Xaa = Gly, Val, Pro
               Pos 4: Xaa = Lys, Val, Arg, Gly, Glu
               Pos 5: Xaa = Ala, Val, Gly, Leu, Ile
               Pos 6: Xaa = Gly, Phe, Trp, Tyr, Val"

     (ix) FEATURE:
          (A) NAME/KEY: Modified-site
          (B) LOCATION:9..18
          (D) OTHER INFORMATION:/product= "Xaa"
               /label= Xaa
               /note= "Pos 10: Xaa = Phe, Tyr, Trp
               Pos 11: Xaa = Leu, Ala, Gly, Ile, Ser, Met
               Pos 12: Xaa = Leu, Ala, Gly, Ile, Ser, Met;
               Pos 13: Xaa = Arg, Lys,
               Pos 18: Xaa = Phe, Tyr

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ser Xaa Xaa Xaa Xaa Xaa Pro Arg Pro Xaa Xaa Xaa Xaa Asn Pro Gly
 1               5                  10                  15

Asp Xaa Glu Glu Ile Pro Glu Glu Tyr Leu Gln
            20                  25


(2) INFORMATION FOR SEQ ID NO: 2:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 12 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS:
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

      (v) FRAGMENT TYPE: C-terminal

     (ix) FEATURE:
          (A) NAME/KEY: Modified-site
          (B) LOCATION:2..4
          (D) OTHER INFORMATION:/product= "Xaa"
               /label= Xaa
               /note= "Pos 3: Xaa = Phe, Tyr"

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gly Asp Xaa Glu Glu Ile Pro Glu Glu Tyr Leu Gln
1               5                  10


(2) INFORMATION FOR SEQ ID NO: 3:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 12 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS:
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

      (v) FRAGMENT TYPE: C-terminal

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Asn Asp Lys Tyr Glu Pro Phe Glu Glu Tyr Leu Gln
```

-continued

1 5 10

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: C-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ser Asp Phe Glu Glu Phe Ser Leu Asp Asp Ile Glu Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: C-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Ser Glu Phe Glu Glu Phe Glu Ile Asp Glu Glu Glu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0105"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TATGAGCAAA ACTTGCTACG AAGGTAACGG TCACTTCTAC CGTGGTAAGG CTTCTACCGA 60

CAC 63

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0106"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

-continued

CATGGTGTCG GTAGAAGCCT TACCACGGTA GAAGTGACCG TTACCTTCGT AGCAAGTTTT 60

GCTCA 65

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 83 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0220"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CGGTTAAGGC TTTCCCGAGG CCTGGTGGTG GTGGTAACGG TGACTTCGAA GAAATCCCGG 60

AAGAGTACCT GTGATAGGAT CAA 83

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0221"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTAGTTGATC CTATCACAGG TACTCTTCCG GGATTTCTTC GAAGTCACCG TTACCACCAC 60

CACCAGGCCT CGGGAAAGCC TTAACCGGGC T 91

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0265"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CACCCGGCGG AGACGGCGGG CTCAGAGCCA GACCGTTTTC TTCTTTGGTG TGAGAACG 58

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

-continued

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0281"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CGTCCGGGTG GTGGTGGTAA CGGTGACTTC GAAGAAATCC CGGAAGAATA CCTGTAAG 58

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0282"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GATCCGTTCT CACACCAAAG AAGAAAACGG TCTGGCTCTG AGCCCGCCGT CTCCGCCGGG 60

TGGTTTCCCG 70

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0283"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CTAGCTTACA GGTATTCTTC CGGGATTTCT TCGAAGTCAC CGTTACCACC ACCACCCGGA 60

CGCGGGAAAC 70

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0329"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

-continued

AAGAAATCCC GGAAGAATAC CTGCAATAAG 30

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0330"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CGGTTAAGGC CTTGGGGACC GCGGCCGCTG GGTGGTGGTG GTAACGGTGA CTTCG 55

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0331"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ACCACCACCC AGCGGCCGCG GTCCCCAAGC CTTAACCGGG CT 42

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0332"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CTAGCTTATT GCAGGTATTC TTCCGGGATT TCTTCGAAGT CACCGTTACC 50

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0347"

(iii) HYPOTHETICAL: NO

-continued

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CGGTTGTTGC TTTCCCGC 18

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0348"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GGCCGCGGGA AAGCAACAAC CGGGCT 26

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0545"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CTAGCTTATT GCAGGTATTC TTCGAACGGT TCGTATTTGT CGTTAGGGTT ACGCAGCAGG 60

AAA 63

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo 0546"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GGCCTTTCCT GCTGCGTAAC CCTAACGACA AATACGAACC GTTCGAAGAA TACCTGCAAT 60

AAC 63

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs

-continued

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo O615"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CTAGCTTATT GCAGGTATTC TTCCGGGATT TCTTCGAAGT CACCAGGGTT ACGCAGCAGG 60

AAA 63

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Synthetic DNA; Nucleotide sequence for Oligo O618"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GGCCTTTCCT GCTGCGTAAC CCTGGTGACT TCGAAGAAAT CCCGGAAGAA TACCTGCAAT 60

AAG 63

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 393 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

```
Met Ser Lys Thr Cys Tyr Glu Gly Asn Gly His Phe Tyr Arg Gly Lys
 1               5                  10                  15

Ala Ser Thr Asp Thr Met Gly Arg Pro Cys Leu Pro Trp Asn Ser Ala
            20                  25                  30

Thr Val Leu Gln Gln Thr Tyr His Ala His Arg Ser Asp Ala Leu Gln
        35                  40                  45

Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Asn Arg Arg
    50                  55                  60

Arg Pro Trp Cys Tyr Val Gln Val Gly Leu Lys Pro Leu Val Gln Glu
65                  70                  75                  80

Cys Met Val His Asp Cys Ala Asp Gly Lys Lys Pro Ser Ser Pro Pro
                85                  90                  95

Glu Glu Leu Lys Phe Gln Cys Gly Gln Lys Thr Leu Arg Pro Arg Phe
            100                 105                 110

Lys Ile Ile Gly Gly Glu Phe Thr Thr Ile Glu Asn Gln Pro Trp Phe
        115                 120                 125

Ala Ala Ile Tyr Arg Arg His Arg Gly Gly Ser Val Thr Tyr Val Cys
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-continued

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    130                 135                 140

Gly Gly Ser Leu Ile Ser Pro Cys Trp Val Ile Ser Ala Thr His Cys
145                 150                 155                 160

Phe Ile Asp Tyr Pro Lys Lys Glu Asp Tyr Ile Val Tyr Leu Gly Arg
                165                 170                 175

Ser Arg Leu Asn Ser Asn Thr Gln Gly Glu Met Lys Phe Glu Val Glu
            180                 185                 190

Asn Leu Ile Leu His Lys Asp Tyr Ser Ala Asp Thr Leu Ala His His
        195                 200                 205

Asn Asp Ile Ala Leu Leu Lys Ile Arg Ser Lys Glu Gly Arg Cys Ala
    210                 215                 220

Gln Pro Ser Arg Thr Ile Gln Thr Ile Cys Leu Pro Ser Met Tyr Asn
225                 230                 235                 240

Asp Pro Gln Phe Gly Thr Ser Cys Glu Ile Thr Gly Phe Gly Lys Glu
                245                 250                 255

Asn Ser Thr Asp Tyr Leu Tyr Pro Glu Gln Leu Lys Met Thr Val Val
            260                 265                 270

Lys Leu Ile Ser His Arg Glu Cys Gln Gln Pro His Tyr Tyr Gly Ser
        275                 280                 285

Glu Val Thr Thr Lys Met Leu Cys Ala Ala Asp Pro Gln Trp Lys Thr
    290                 295                 300

Asp Ser Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys Ser Leu Gln
305                 310                 315                 320

Gly Arg Met Thr Leu Thr Gly Ile Val Ser Trp Gly Arg Gly Cys Ala
                325                 330                 335

Leu Lys Asp Lys Pro Gly Val Tyr Thr Arg Val Ser His Phe Leu Pro
            340                 345                 350

Trp Ile Arg Ser His Thr Lys Glu Glu Asn Gly Leu Ala Leu Ser Pro
        355                 360                 365

Val Val Ala Phe Pro Arg Pro Phe Leu Leu Arg Asn Pro Gly Asp Phe
    370                 375                 380

Glu Glu Ile Pro Glu Glu Tyr Leu Gln
385                 390
```

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 393 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

```
Met Ser Lys Thr Cys Tyr Glu Gly Asn Gly His Phe Tyr Arg Gly Lys
1               5                   10                  15

Ala Ser Thr Asp Thr Met Gly Arg Pro Cys Leu Pro Trp Asn Ser Ala
            20                  25                  30

Thr Val Leu Gln Gln Thr Tyr His Ala His Arg Ser Asp Ala Leu Gln
        35                  40                  45

Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Asn Arg Arg
    50                  55                  60

Arg Pro Trp Cys Tyr Val Gln Val Gly Leu Lys Pro Leu Val Gln Glu
65                  70                  75                  80

Cys Met Val His Asp Cys Ala Asp Gly Lys Lys Pro Ser Ser Pro Pro
```

-continued

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                85                  90                  95

Glu Glu Leu Lys Phe Gln Cys Gly Gln Lys Thr Leu Arg Pro Arg Phe
            100                 105                 110

Lys Ile Ile Gly Gly Glu Phe Thr Thr Ile Glu Asn Gln Pro Trp Phe
        115                 120                 125

Ala Ala Ile Tyr Arg Arg His Arg Gly Gly Ser Val Thr Tyr Val Cys
    130                 135                 140

Gly Gly Ser Leu Ile Ser Pro Cys Trp Val Ile Ser Ala Thr His Cys
145                 150                 155                 160

Phe Ile Asp Tyr Pro Lys Lys Glu Asp Tyr Ile Val Tyr Leu Gly Arg
                165                 170                 175

Ser Arg Leu Asn Ser Asn Thr Gln Gly Glu Met Lys Phe Glu Val Glu
            180                 185                 190

Asn Leu Ile Leu His Lys Asp Tyr Ser Ala Asp Thr Leu Ala His His
        195                 200                 205

Asn Asp Ile Ala Leu Leu Lys Ile Arg Ser Lys Glu Gly Arg Cys Ala
    210                 215                 220

Gln Pro Ser Arg Thr Ile Gln Thr Ile Cys Leu Pro Ser Met Tyr Asn
225                 230                 235                 240

Asp Pro Gln Phe Gly Thr Ser Cys Glu Ile Thr Gly Phe Gly Lys Glu
                245                 250                 255

Asn Ser Thr Asp Tyr Leu Tyr Pro Glu Gln Leu Lys Met Thr Val Val
            260                 265                 270

Lys Leu Ile Ser His Arg Glu Cys Gln Gln Pro His Tyr Tyr Gly Ser
        275                 280                 285

Glu Val Thr Thr Lys Met Leu Cys Ala Ala Asp Pro Gln Trp Lys Thr
    290                 295                 300

Asp Ser Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys Ser Leu Gln
305                 310                 315                 320

Gly Arg Met Thr Leu Thr Gly Ile Val Ser Trp Gly Arg Gly Cys Ala
                325                 330                 335

Leu Lys Asp Lys Pro Gly Val Tyr Thr Arg Val Ser His Phe Leu Pro
            340                 345                 350

Trp Ile Arg Ser His Thr Lys Glu Glu Asn Gly Leu Ala Leu Ser Pro
        355                 360                 365

Val Val Ala Phe Pro Arg Pro Phe Leu Leu Arg Asn Pro Asn Asp Lys
    370                 375                 380

Tyr Glu Pro Phe Glu Glu Tyr Leu Gln
385                 390
```

What is claimed is:

1. A chimeric protein with fibrinolytic and thrombin-inhibiting properties comprising a plasminogen-activating amino acid sequence which is linked at its C-terminal end to an amino acid sequence of the formula I

$$Ser\text{-}X_1\text{-}X_2\text{-}X_3\text{-}X_4\text{-}X_5\text{-}Pro\text{-}Arg\text{-}Pro\text{-}Y_1\text{-}Y_2\text{-}Y_3\text{-}Y_4\text{-}Asn\text{-}Pro\text{-}Z \quad (I)$$

(SEQ ID NO: 1),
wherein

X_1 represents Pro or Leu;

X_2 represents Gly, Val or Pro;

X_3 represents Lys, Val, Arg, Gly or Glu;

X_4 represents Ala, Val, Gly, Leu or Ile;

X_5 represents Gly, Phe, Trp, Tyr or Val;

Y_1 represents Phe;

Y_2 represents Leu;

Y_3 represents Leu;

Y_4 represents Arg, and

Z represents at least one amino acid sequence selected from the group consisting of:

$$Gly\text{-}Asp\text{-}Z_1\text{-}Glu\text{-}Glu\text{-}Ile\text{-}Pro\text{-}Glu\text{-}Glu\text{-}Tyr\text{-}Leu\text{-}Gln \quad (II)$$

(SEQ ID NO: 2),
wherein Z_1 represents Phe or Tyr,

$$Asn\text{-}Asp\text{-}Lys\text{-}Tyr\text{-}Glu\text{-}Pro\text{-}Phe\text{-}Glu\text{-}Glu\text{-}Tyr\text{-}Leu\text{-}Gln \quad (III)$$

(SEQ ID NO: 3),

$$Ser\text{-}Asp\text{-}Phe\text{-}Glu\text{-}Glu\text{-}Phe\text{-}Ser\text{-}Leu\text{-}Asp\text{-}Asp\text{-}Ile\text{-}Glu\text{-}Gln \quad (IV)$$

(SEQ ID NO: 4), and

Ser-Glu-Phe-Glu-Glu-Phe-Glu-Ile-Asp-Glu-Glu-Glu-Lys (V)

(SEQ ID NO: 5),

said plasminogen-activating sequence being selected from the group consisting of:

the unaltered amino acid sequence of prourokinase,

modified prourokinase amino acid sequences having a serine protease domain exhibiting at least 95% sequence identity to the serine protease domain of the unaltered amino acid sequence of prourokinase,

the unaltered amino acid sequence of urokinase,

modified urokinase amino acid sequences having a serine protease domain exhibiting at least 95% sequence identity to the serine protease domain of the unaltered amino acid sequence of urokinase,

the unaltered amino acid sequence of tissue plasminogen activator (t-PA), and

modified t-PA amino acid sequences having a serine protease domain exhibiting at least 95% sequence identity to the serine protease domain of the unaltered amino acid sequence of t-PA.

2. A protein according to claim 1, wherein the plasminogen-activating amino acid sequence comprises at least one amino acid sequence which includes a serine protease domain and is selected from the group consisting of:

the unaltered amino acid sequence of prourokinase,

modified prourokinase amino acid sequences having a serine protease domain exhibiting at least 95% sequence identity to the serine protease domain of the unaltered amino acid sequence of prourokinase,

the unaltered amino acid sequence of t-PA, and

modified t-PA amino acid sequences having a serine protease domain exhibiting at least 95% sequence identity to the serine protease domain of the unaltered amino acid sequence of t-PA.

3. A protein according to claim 2, wherein the plasminogen-activating amino acid sequence comprises at least one sequence selected from the group consisting of:

the amino acid sequence of prourokinase consisting of 411 amino acids in which the amino acid in position 407 is Asn or Gln,

the ^{47}Ser to ^{421}Leu amino acid sequence of prourokinase in which the amino acid in position 407 is Asn or Gln,

the ^{138}Ser to ^{413}Leu amino acid sequence of prourokinase in which the amino acid in position 407 is Asn or Gln,

the unaltered amino acid sequence of t-PA consisting of 527 amino acids,

the Ser-^{89}Arg to 527Pro amino acid sequence of t-PA, and

the ^{174}Ser to ^{527}Pro amino acid sequence of t-PA.

4. A protein according to claim 1, wherein in the amino acid sequence of formula I, X_1 represents Pro; X_2 represents Val; X_3 represents Lys or Val; X_4 represents Ala, and X_5 represents Phe.

5. A protein according to claim 1, wherein in the amino acid sequence of formula I, Z represents an amino acid sequence of formula II or an amino acid sequence of formula IV.

6. A chimeric protein with fibrinolytic and thrombin-inhibiting properties comprising a plasminogen-activating amino acid sequence which is linked at its C-terminal end to an amino acid sequence of the formula I

Ser-X_1-X_2-X_3-X_4-X_5-Pro-Arg-Pro-Y_1-Y_2-Y_3-Y_4-Asn-Pro-Z (I)

(SEQ ID NO: 1),

wherein

X_1 represents Pro or Leu;

X_2 represents Gly, Val or Pro;

X_3 represents Lys, Val, Arg, Gly or Glu;

X_4 represents Ala, Val, Gly, Leu or Ile;

X_5 represents Gly, Phe, Trp, Tyr or Val;

Y_1 represents Phe;

Y_2 represents Leu;

Y_3 represents Leu;

Y_4 represents Arg; and

Z represents at least one amino acid sequence selected from the group consisting of:

Gly-Asp-Z_1-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln (II)

(SEQ ID NO: 2), wherein Z_1 represents Phe or Tyr,

Asn-Asp-Lys-Tyr-Glu-Pro-Phe-Glu-Glu-Tyr-Leu-Gln (III)

(SEQ ID NO: 3),

Ser-Asp-Phe-Glu-Glu-Phe-Ser-Leu-Asp-Asp-Ile-Glu-Gln (IV)

(SEQ ID NO: 4), and

Ser-Glu-Phe-Glu-Glu-Phe-Glu-Ile-Asp-Glu-Glu-Glu-Lys (V)

(SEQ ID NO: 5),

said plasminogen-activating sequence comprising at least one amino acid sequence selected from the group consisting of:

an unaltered amino acid sequence of prourokinase,

a modified prourokinase amino acid sequence having at least 95% sequence identity to the unaltered amino acid sequence of prourokinase,

an unaltered amino acid sequence of t-PA, and

a modified t-PA amino acid sequence having at least 95% sequence identity to the unaltered amino acid sequence of t-PA.

7. A thrombolytic composition comprising a chimeric protein according to claim 1, and at least one conventional pharmaceutical carrier or adjuvant.

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